

Coordinated regulation of chemotaxis and resistance to copper by CsoR in *Pseudomonas putida*

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eLife Assessment

Data presented in this **useful** report suggest a potentially new model for chemotaxis regulation in the gram-negative bacterium *P. putida*. Data supporting interactions between CheA and the copper-binding protein CsoR, reveal potential mechanisms for coordinating chemotaxis and copper resistance. There was, however, concern about the large number of CheA interactors identified in the initial screen and it was felt that the study was **incomplete** without a substantial number of additional experiments to test the model and bolster the authors' conclusions.

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Abstract

Copper is an essential enzyme cofactor in bacteria, but excess copper is highly toxic. Bacteria can cope with copper stress by increasing copper resistance and initiating chemorepellent response. However, it remains unclear how bacteria coordinate chemotaxis and resistance to copper. By screening proteins that interacted with the chemotaxis kinase CheA, we identified a copper-binding repressor CsoR that interacted with CheA in *Pseudomonas putida*. CsoR interacted with the HPT (P1), Dimer (P3), and HATPase_c (P4) domains of CheA and inhibited CheA autophosphorylation, resulting in decreased chemotaxis. The copper-binding of CsoR weakened its interaction with CheA, which relieved the inhibition of chemotaxis by CsoR. In addition, CsoR bound to the promoter of copper-resistance genes to inhibit gene expression, and copper-binding released CsoR from the promoter, leading to increased gene expression and copper resistance. *P. putida* cells exhibited a chemorepellent response to copper in a CheA-dependent manner, and CsoR inhibited the chemorepellent response to copper. Besides, the CheA-CsoR interaction also existed in proteins from several other bacterial species. Our results revealed a mechanism by which bacteria coordinately regulated chemotaxis and resistance to copper by CsoR.

Introduction

Chemotaxis is a widespread ability of motile bacteria to direct their movement towards higher concentrations of beneficial chemicals or lower concentrations of toxic chemicals^{1,2}. Chemotaxis plays a vital role in bacterial exploration and adaptation to complex environments^{3,4}. The chemotaxis signaling pathway is extensively studied in the enteric bacteria *Escherichia coli* and *Salmonella enterica* serovar Typhimurium^{2,5}. In *E. coli*, the chemotaxis system consists of five methyl-accepting chemotaxis proteins (MCPs) and six core components (the kinase CheA, the response regulator CheY, the coupling protein CheW, the methyltransferase CheB, the phosphatase CheZ, and the methyltransferase CheR). In response to decreased attractant or increased repellent, methylated MCPs and the coupling protein CheW activate CheA autophosphorylation^{6,7}. The phosphorylated CheA (CheA-P) is a phosphodonator for the response regulator CheY and the methyltransferase CheB. After CheY accepts the phosphate group from CheA, it binds FlhM and FlhN of the motor-switch complex, resulting in a switch in the flagellar rotational direction and bacteria tumbling⁸. Meanwhile, on a slower timescale, phosphorylated CheB demethylates the active MCPs, thus reducing their ability to activate CheA, leading to decreased levels of phosphorylated CheY and less tumbling⁹. The phosphatase CheZ contributes to signal termination by removing the phosphoryl group from phosphorylated CheY, and the methyltransferase CheR contributes to signal adaptation by catalyzing the methylation of MCPs^{2,5}.

Among the bacterial chemotaxis system components, the kinase CheA is a five-domain enzyme central to the chemotaxis signaling pathway^{10,11}. The five domains (P1-P5) of CheA each have distinct functions. The P1 domain (HPT domain) contains the phosphoryl-accepting histidine that becomes phosphorylated, the P2 domain (CheY-binding domain) docks the response regulator proteins CheY and CheB, the P3 domain (Dimer domain) dimerizes the CheA protein, the P4 domain (HATPase_c domain) binds ATP and catalyzes phosphoryl transfer to the histidine residue on P1, and the P5 domain (CheW-binding domain) couples CheA to other chemotaxis components by binding both CheW and the chemoreceptors^{10,11}. Except for interacting with the components in chemotaxis system, CheA is reported to interact with proteins from other systems. For example, in the plant pathogen *Xanthomonas oryzae* pv. *oryzicola*, CheA interacts with and phosphorylates the response regulator VemR to regulate bacterial virulence, motility, and EPS production¹². In *Vibrio parahaemolyticus*, a polarly localized protein ParP interacts with CheA and prevents its dissociation from chemotaxis signaling arrays, facilitating proper chemotaxis and accurate inheritance of these macromolecular chemotactic machines¹³. In *Comamonas testosteroni*, CheA interacts with and phosphorylates the response regulator FlmD, resulting in decreased biofilm formation¹⁴. In *Azospirillum brasilense* with two chemotaxis signaling systems, the kinase CheA from each chemotaxis signaling system physically interacts with the CheY response regulator of another system¹⁵. In *Pseudomonas aeruginosa*, CheA interacts with the phosphodiesterase DipA to regulate its subcellular localization and activity, leading to individual cell heterogeneity and motility behavior diversity in bacterial populations¹⁶. These studies suggest that the CheA-mediated crosstalk between chemotaxis and other systems coordinates complex behaviors in diverse bacteria.

In most living organisms, copper is an essential cofactor for enzymes involved in fundamental processes such as respiration and photosynthesis^{17,18}. However, copper also has toxic effects on cells, and bacteria have several strategies to increase their resistance to copper^{19–21}. The direct bacterial response associated with copper resistance is highly conserved and generally involves (a) sensing of the increased copper concentration by sensors, (b) activation of bespoke transcriptional networks, (c) overproduction of copper efflux pumps that secrete copper out of the cells, and (d) recruitment of copper-binding and copper-oxidizing proteins that prevent copper from interacting with cellular components^{22–26}. The genome of *Pseudomonas putida* was

predicated to encode a dozen of proteins involved in copper resistance²⁷, including copper sensor proteins (CopS-I, CopS-II), P-type ATPases for copper efflux (CueA, CopA-I, CopA-II, CopB-I, and CopB-II), heavy-metal efflux complex components (CusA, CusB, CusC, and CusF). Expression of these proteins were regulated by copper-responsive positive regulatory proteins (CopR-I, CopR-II, CueR)^{28,29}. CueR regulated the expression of genes implicated in cytoplasmic copper homeostasis, whereas CopR controlled the expression of genes involved in maintaining periplasmic metal level³⁰. Meanwhile, some proteins/systems appeared to be duplicated, and some were proved to be functionally redundant^{28,30}. Besides, extracellular polymeric substances (EPS) in *P. putida* biofilms showed high affinity for most heavy metals, including copper, thereby providing a protective barrier under copper stress^{31–33}.

In addition to the above copper resistance strategies, bacteria can avoid copper stress through chemotaxis. For example, in *Caulobacter crescentus*, the reactive oxygen species derived from cytoplasmic copper ions mediate the bacterial chemotaxis to copper, and a potential cytoplasmic MCP McpR regulates bacterial chemotaxis in response to cellular copper content, enabling bacteria to escape from copper-rich environment^{34,35}. However, unlike the widely reported mechanisms of copper resistance in diverse bacterial species, the mechanism(s) of bacterial chemotaxis to copper is poorly studied. Besides, it remains unclear how bacteria coordinate chemotaxis and resistance to copper.

Since the kinase CheA plays a central role in chemotaxis signaling, identifying CheA-interacting proteins would extend the knowledge of chemotaxis regulation. In this study, by screening proteins that interacted with CheA in *Pseudomonas putida*, we obtained 16 novel CheA-interacting proteins. Among the 16 proteins, CsoR, a copper-binding transcription regulator, inhibited the autophosphorylation of CheA, leading to decreased chemotaxis. Meanwhile, CsoR functioned as a DNA-binding repressor to inhibit the expression of copper-resistance genes. Copper-binding of CsoR relieved its inhibition of gene expression and chemotaxis.

Results

Identification of new CheA-interacting proteins in *P. putida*

We performed a pull-down assay to identify protein(s) interacting with CheA. Purified 6 × His-tagged CheA bound onto a Ni-NTA agarose column was used as “bait” protein to pull out potential CheA-interacting “prey” protein(s) from whole cell lysate of *P. putida* KT2440, and a blank Ni-NTA agarose column was used as a negative control. All “prey” proteins from the CheA-binding column and control column were collected, resolved by SDS-PAGE (Fig. 1a), and analyzed by mass spectrometry (MS). In MS analysis, 43 proteins showed a significantly higher amount in the CheA-binding column than in the control column ($\text{Log}_2(\text{fold change}) > 2$) (Fig. 1b, Table S1). As expected, the “bait” protein CheA showed the highest amount, with a $\text{Log}_2(\text{fold change})$ value close to 8. Meanwhile, the response regulator CheY and the phosphatase CheZ (two proteins known to be associated with CheA) also showed high $\text{Log}_2(\text{fold change})$ values. In addition to these three proteins (CheA, CheY, and CheZ), the remaining 40 proteins were considered potential new CheA-interacting proteins.

To verify the above result, we performed bacterial two-hybrid (BTH) assay to test the interactions between CheA and the 40 proteins. The results from the BTH assay revealed that 19 proteins showed apparent interaction with CheA, including PP_2969 (CsoR), PP_1612 (Eno), PP_4111 (FusB), PP_5046 (GlnA), PP_1074 (GlpR), PP_4728 (GrpE), PP_0853 (IspG), PP_1877 (MsrC), PP_2378 (NfuA), PP_1023 (Pgl), PP_5006 (PhaD), PP_0691 (ProB), PP_0148, PP_1644, PP_2683, PP_3177, PP_3227, PP_3501, and PP_4460 (Fig. 1c). Meanwhile, the rest 21 proteins displayed no significant interaction with CheA in BTH assay (Fig. S1a and S1b), suggesting that they were false positive results in the pull-down assay. We further tested the interactions between CheA and the 19

proteins in *P. putida* using bimolecular fluorescence complementation (BiFC) assay. The results displayed that except three proteins (PP_2683, PP_3227, and PP_4460) showed no apparent interaction with CheA, the other 16 proteins displayed obvious interaction with CheA in the BiFC assay (**Fig. 1d**, Fig. S2). Collectively, using pull-down, BTH, and BiFC assays, we identified 16 new CheA-interacting proteins in *P. putida*, including CsoR, Eno, FusB, GlnA, GlpR, GrpE, IspG, MsrC, NfuA, Pgl, PhaD, ProB, PP_0148, PP_1644, PP_3177, and PP_3501.

Effect of CheA-interacting proteins on bacterial chemotaxis

Since CheA played a crucial role in bacterial chemotaxis, we wondered whether the 16 CheA-interacting proteins were involved in regulating chemotaxis. Thus, we overexpressed each of the 16 proteins in wild-type *P. putida* and tested the chemotaxis ability. The wild-type *P. putida* harboring empty vector (WT+pVec) was used as the control. Chemotaxis ability was assessed using semisolid nutrient agar plates on which bacteria formed large colonies (swimming zone) by generating and following attractant gradients leading outward from the colony origin³⁶. Growth of the 16 strains in liquid medium showed a similar trend as that of the control strain (Fig. S3), suggesting that the 16 proteins had no noticeable effect on bacterial growth. As shown in **Fig. 2a**, five strains (WT+pcsoR, WT+pispG, WT+pnfuA, WT+pphaD, and WT+pPP_1644) displayed smaller colony than the control strain (WT+pVec), indicating a weaker chemotaxis ability in these five strains. The other 11 strains showed a similar chemotaxis ability as WT+pVec. These results suggested that five CheA-interacting proteins (CsoR, IspG, NfuA, PhaD, and PP_1644) inhibited chemotaxis in *P. putida*.

CsoR and PhaD inhibit CheA autophosphorylation

CheA has autophosphorylation activity, and it can phosphorylate its cognate response regulator¹¹. To test whether phosphate transfer existed between CheA and the 16 proteins, we performed a phosphate transfer assay using purified proteins and [³²P]ATP[γP]. We successfully purified 14 out of the 16 proteins but failed to purify two proteins (GlpR and PP_3177) after several attempts. Thus, the two proteins were not included in the phosphate transfer assay. The results showed that CheA exhibited a strong autophosphorylation signal after incubation with [³²P]ATP[γP] for 45 min (Fig. S4a). Then, each of the 14 proteins or CheY (positive control) was added to phosphorylated CheA to investigate the phosphate transfer. The addition of CheY to phosphorylated CheA led to a labeling of CheY and a reduction in the phospholabeling of CheA (Fig. S4a), indicating a phosphate transfer happened between CheA and CheY. However, no labeling of the 14 target proteins was observed, and there was no apparent change in the phospholabeling of CheA after adding each of the 14 proteins (Fig. S4a), suggesting no phosphate transfer happened between CheA and the 14 tested proteins.

Then, we wondered whether the 14 proteins influenced CheA autophosphorylation. To answer this question, we mixed CheA with each of the 14 proteins before adding the substrate [³²P]ATP[γP]. The mixture containing CheA and bovine serum albumin (BSA) was used as a negative control. As shown in **Fig. 2b**, CsoR/PhaD significantly decreased the phospholabeling of CheA. In contrast, the other 12 proteins and BSA had no apparent influence on the phospholabeling of CheA. We further tested the impact of CsoR/PhaD on CheA autophosphorylation with a more detailed assay, in which CheA was mixed with an increased amount of CsoR/PhaD. The results showed that CheA phospholabeling decreased as CsoR/PhaD increased. In contrast, the increase of BSA had no obvious influence on CheA phospholabeling (**Fig. 2c** and **2d**). These results indicated that CsoR and PhaD inhibited CheA autophosphorylation. It was also possible that CsoR and PhaD degraded the substrate [³²P]ATP[γP] in the reaction mixture, resulting in decreased CheA autophosphorylation. To test this possibility, we examined whether CsoR and PhaD had ATPase activity. The known ATPase FleQ was used as a positive control, and BSA was used as a negative control. The results showed that adding FleQ into the reaction mixture caused a decrease in ATP

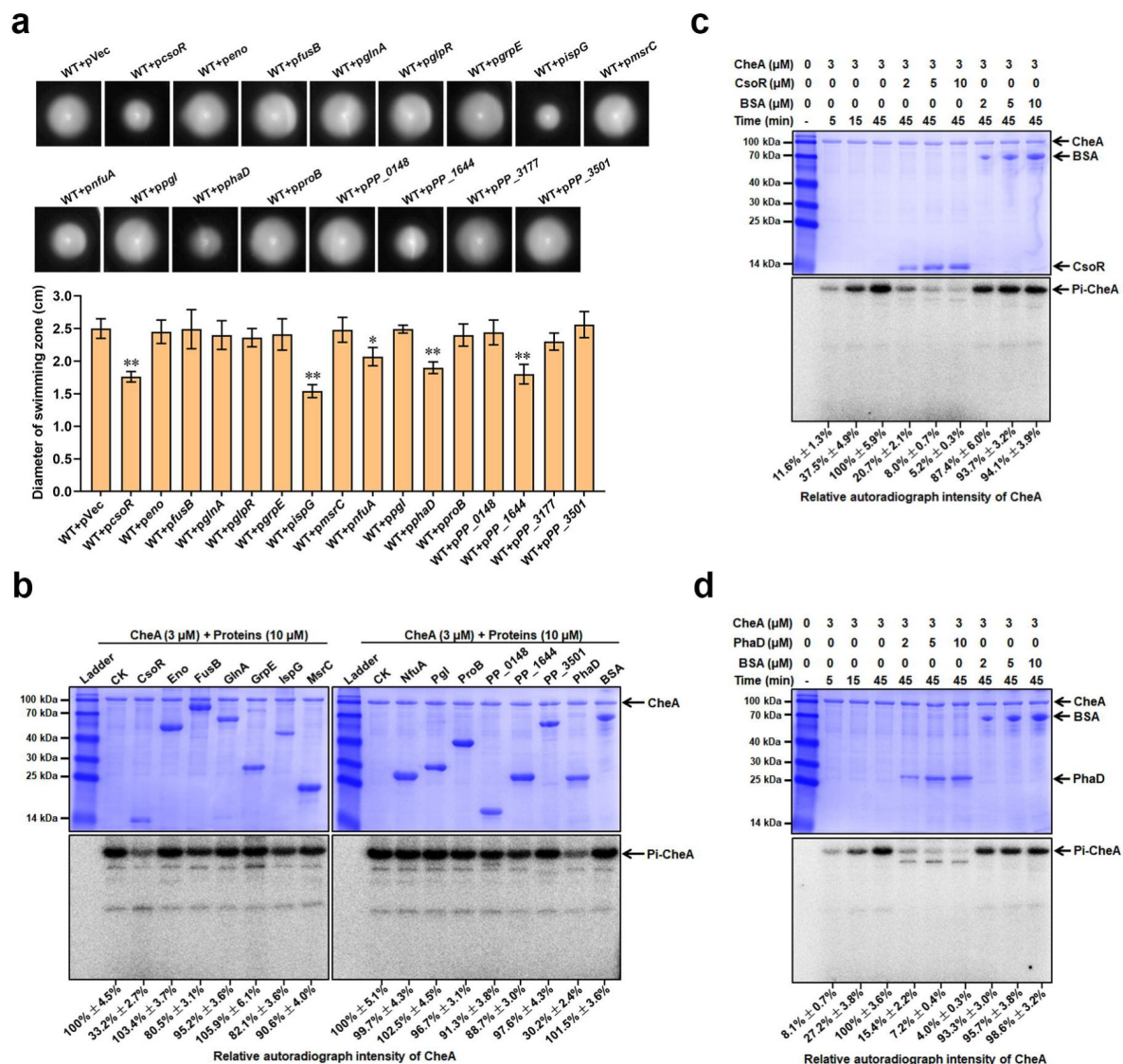


Fig. 2

CsoR and PhaD inhibit CheA autophosphorylation.

(a) Chemotaxis of indicated strains on semisolid plates. Photos of colonies on the top were taken after 16 h incubation at 28°C. Diameters of colonies (swimming zone) shown at down were calculated from three replicates. The asterisks above the column denote significant differences ($*P < 0.05$, $**P < 0.01$) between indicated strains and control strain (WT+pVec) analyzed by Student's t-test. (b) Effect of the 14 proteins on the CheA autophosphorylation. The name/ID and the concentration of tested proteins added in each lane are indicated above the gel. CK represents CheA alone in the reaction mixture. BSA is used as a negative control. The ladder represents a protein marker. (c and d) CsoR (c) and PhaD (d) affect CheA autophosphorylation. The concentration of tested proteins added in each lane is indicated above the gel. The time represents the time of the CheA autophosphorylation reaction. The SDS-PAGE gels in panels b, c, and d were detected by Coomassie Blue Staining (Above) and autoradiograph (Below). The experiments for panels b, c, and d were repeated three times, and a representative photo was shown. The relative autoradiograph intensity of the CheA band was calculated using Image J software and shown below each lane.

level (Fig. S4b), indicating the existence of ATPase activity. Meanwhile, the addition of CsoR/PhaD exhibited no apparent influence on ATP level as the addition of BSA (Fig. S4b), suggesting that CsoR and PhaD had no ATPase activity.

In the chemotaxis signaling pathway, CheA transfers the phosphate group to the response regulator CheY to modulate flagellar rotation^{2,5}. Since CsoR and PhaD interacted with CheA, we wondered whether CsoR/PhaD influenced the phosphate transfer between CheA and CheY. Thus, we incubated CsoR/PhaD with the phosphorylated CheA before adding CheY to the reaction mixture. The results revealed that CheA phospholabeling in the CsoR/PhaD-adding group reduced to a similar level as that in the group without CsoR/PhaD (Fig. S4c), implying that CsoR/PhaD had no apparent influence on the phosphate transfer between CheA and CheY.

The domains of CheA involved in interacting with CsoR and PhaD

To further explore how CsoR and PhaD affect CheA autophosphorylation, we determined the domain(s) of CheA involved in interacting with CsoR and PhaD. Similar to the *E. coli* CheA, the *P. putida* CheA consists of five domains with distinct functions (Fig. 3a). We constructed five truncated CheA variants, with each missing one domain (termed CheA_{ΔHPT}/CheA_{ΔYB}/CheA_{ΔDim}/CheA_{ΔHATPase}/CheA_{ΔWB}) (Fig. 3a). Then we performed BTH assay to test the interaction between CsoR/PhaD and each of these truncated CheAs. The results revealed that three truncated CheAs (CheA_{ΔHPT}, CheA_{ΔDim}, and CheA_{ΔHATPase}) showed no interaction with CsoR and PhaD. In comparison, the other two truncated CheAs (CheA_{ΔYB} and CheA_{ΔWB}) interacted with CsoR and PhaD with a similar intensity as the wild-type CheA did (Fig. 3b and 3c), indicating that the HPT (P1), Dimer (P3), and HATPase_c (P4) domains were essential for interacting with CsoR and PhaD, while the YB (P2) and WB (P5) domains were not required to interact with CsoR and PhaD. To further test this result, we cloned each of the five domains into a BTH vector and tested the interaction between CsoR/PhaD and each of the five domains. The result showed that the Dimer domain (Dim_{CheA}) interacted with CsoR and PhaD like the whole-length CheA did (Fig. 3b and 3c). The HPT domain (HPT_{CheA}) and the HATPase_c domain (HATPase_{CheA}) showed a weaker interaction with CsoR and PhaD. Meanwhile, the YB domain (YB_{CheA}) and the WB domain (WB_{CheA}) displayed no interaction with CsoR and PhaD (Fig. 3b and 3c). Together, these results revealed that the Dimer domain (P3) of CheA played a significant role, the HPT (P1) and HATPase_c (P4) domains played a minor role, while the CheY-binding (P2) and CheW-binding (P5) domains played no role in the interaction between CheA and CsoR/PhaD. Since the three domains involved in interacting with CsoR/PhaD were also essential for CheA autophosphorylation activity^{10,11}, CsoR/PhaD might inhibit CheA autophosphorylation by inhibiting the function of the three domains.

Of the two proteins that inhibit CheA autophosphorylation, CsoR is annotated as a metal-binding transcriptional repressor, and PhaD is annotated as a TetR family transcriptional regulator³⁷. Although the two proteins were quite different in size (CsoR 10.8 kDa, PhaD 23.1 kDa), they interacted with the same domains of CheA to inhibit its autophosphorylation, we wondered whether the two proteins shared similarity in sequence and structure. BLAST result revealed no sequence similarity between CsoR and PhaD (data not shown). We predicted structures of CsoR and PhaD using AlphaFold (Fig. S5a and S5b), and compared their structures using Pymol. However, the result revealed no significant structural similarity between the two proteins (Fig. S5c).

CsoR is a metal-binding transcriptional repressor for metal-resistance genes

Previous research revealed that the expression of CsoR (also named MreA) was induced by cadmium, nickel, zinc, and cobalt³⁸. The homolog of CsoR in *Mycobacterium tuberculosis* binds to copper and regulates the expression of copper-resistance genes^{39,40}. However, the metal-binding ability and the function of the *P. putida* CsoR were not experimentally characterized. Thus,

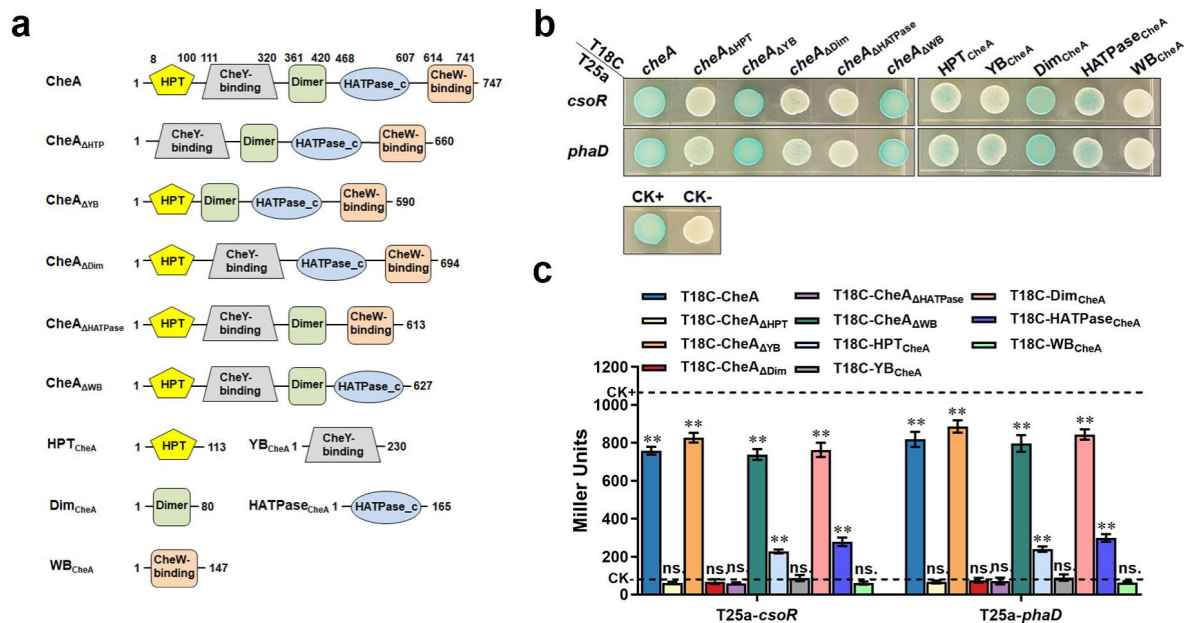


Fig. 3

CheA domains involved in interacting with CsoR and PhaD.

(a) Schematic diagram of CheA and truncated CheA proteins. The predicted domains are based on the Pfam database and the amino acid positions where the predicted domains start and end are shown. (b) The interaction between CheA domains and CsoR/PhaD was tested using BTH. Blue indicates protein-protein interaction in the colony after 60 h of incubation, while white indicates no protein-protein interaction. A colony containing T25a-zip and T18C-zip plasmids was used as a positive control (CK+), and a colony containing empty T25a and T18C plasmids was used as a negative control (CK-). (c) Confirmation of BTH interactions in panel B by LacZ activity assay. The results are the average of three independent assays. Error bars represent standard deviations. The asterisks above the column denote significant differences (** $P < 0.01$) between indicated strains and CK-strain analyzed by Student's t-test. "ns." represents none statistically significant between indicated strain and CK-strain.

we tested the metal-binding ability of the *P. putida* CsoR using MicroScale Thermophoresis (MST). Six metal ions were involved in the assay, including copper (Cu^{2+}), zinc (Zn^{2+}), nickel (Ni^{2+}), cobalt (Co^{2+}), cadmium (Cd^{2+}), and magnesium (Mg^{2+}). The results revealed that CsoR bound to three out of the six tested metal ions (Cu^{2+} , Zn^{2+} , Ni^{2+}), and the binding to Cu^{2+} was the strongest with a calculated binding constant (K_d) of $5.5 \pm 1.98 \mu\text{M}$. In contrast, the binding to $\text{Ni}^{2+}/\text{Zn}^{2+}$ was weak (with K_d value of $125 \pm 38 \mu\text{M}$ and $253 \pm 42 \mu\text{M}$, respectively) (Fig. 4a). Meanwhile, CsoR showed no apparent binding to $\text{Co}^{2+}/\text{Cd}^{2+}/\text{Mg}^{2+}$ under the same condition (Fig. 4a). We further constructed a *csoR* deletion mutant (ΔcsoR) and investigated the effect of *csoR* deletion on the expression of copper resistance genes using quantitative real-time PCR (qRT-PCR). Three key copper resistance genes (*copA-I*, *copA-II*, and *copB-II*) from two operons were chosen as targets in qRT-PCR. The results showed that *csoR* deletion (ΔcsoR +pVec) led to a weak but significant increase (about 1.3-fold) in the expression of the three genes, and complementation (ΔcsoR +*pcsoR*) decreased the expression of the three genes (Fig. 4b). The addition of CuCl_2 (final concentration $10 \mu\text{M}$) induced the expression of the three genes (about 5-fold) (Fig. 4b). However, no noticeable difference in gene expression level was observed between WT+pVec and ΔcsoR +pVec, as well as between ΔcsoR +pVec and ΔcsoR +*pcsoR* (Fig. 4b), implying that CsoR was not required for the copper-induced genes expression. Besides, the deletion of *cheA* (ΔcheA +pVec) displayed no obvious effect on the expression of the three genes in either the presence or absence of copper (Fig. 4b). Since CsoR also bound Zn^{2+} and Ni^{2+} , we investigated the influence of CsoR on the expression of several other metal resistance genes, including three nickel-resistance genes (*nikA*, *nikB*, and *nikD*), one zinc-resistance genes (*znuC*), two cadmium-resistance genes (*cadA-I* and *cadA-III*), two cobalt-resistance gene (*cbiD* and *cbtA*), and three multiple metal-resistance genes (*czcC-I*, *czcB-II*, and *PP_0026*). The results revealed that CsoR repressed the expression of *nikB*, *cadA-I*, *cadA-III*, *cbtA*, *czcC-I*, *czcB-II*, and *PP_0026*, and the inhibition degree was close to the inhibition degree of copper resistance genes (Fig. S6a). Together, these results demonstrated that CsoR functioned as a metal-binding repressor for metal resistance genes in *P. putida*.

Previous studies reported that CsoR bound the promoter of target genes to inhibit gene expression^{39,40}. We further tested the interaction between CsoR and the promoter of *copA-I* using electrophoretic mobility shift assay (EMSA). The fragment of *copA-I* promoter exhibited a stepwise increase in the shifted DNA amount (CsoR-DNA complex), with the CsoR protein amount increasing from 5 to $20 \mu\text{M}$ (Fig. 4c). Adding Cu^{2+} to the reaction mixture decreased the CsoR-DNA complex (Fig. 4c), indicating that Cu^{2+} inhibited the interaction between CsoR and promoter DNA. Previous study in *M. tuberculosis* showed that CsoR bound a single-monomer mole equivalent of Cu^+ to form a trigonally coordinated complex³⁹, but our results indicated that CsoR bound to Cu^{2+} . To further test whether Cu^+ bind to CsoR and affect its DNA-binding ability, we added dithiothreitol (DTT) to the EMSA reaction mixture. DTT can reduce Cu^{2+} to Cu^+ in solution⁴¹. As shown in the following Fig. 4d, the addition of DTT (0.1 and 1 mM) decreased CsoR-DNA interaction in the presence of 0.2 mM Cu^{2+} , and the addition of DTT alone had no apparent influence on CsoR-DNA interaction, indicating that DTT enhanced the inhibition of Cu^{2+} on CsoR-DNA interaction. These results suggested that the Cu^+ converted from Cu^{2+} by DTT had stronger inhibitory effect than Cu^{2+} on CsoR-DNA interaction, indicating that CsoR bound more strongly to Cu^+ than to Cu^{2+} . Besides, Ni^{2+} , Zn^{2+} , and Cd^{2+} also exhibited an inhibitory effect on the interaction between CsoR and promoter DNA, but to a much lower extent compared with Cu^{2+} (Fig. 4e). Meanwhile, Co^{2+} and Mg^{2+} displayed no obvious effect on the CsoR-DNA interaction (Fig. 4e). These results showed that CsoR was a promoter-binding transcriptional repressor, and binding to metal (especially copper) decreased the interaction between CsoR and promoter DNA.

To test the effect of CsoR and CheA on bacterial copper resistance, we tested the growth of ΔcsoR and ΔcheA under different copper concentrations using both solid agar plate and liquid medium. However, in both cases, there was no significant difference between the growth trend of $\Delta\text{csoR}/\Delta\text{cheA}$ and the WT strain at different copper concentrations (Fig. S6b and S6c). This might attribute to the fact that CsoR was a repressor, and the expression of copper resistance genes in WT was similar to that in ΔcsoR under copper stress (Fig. 4b).

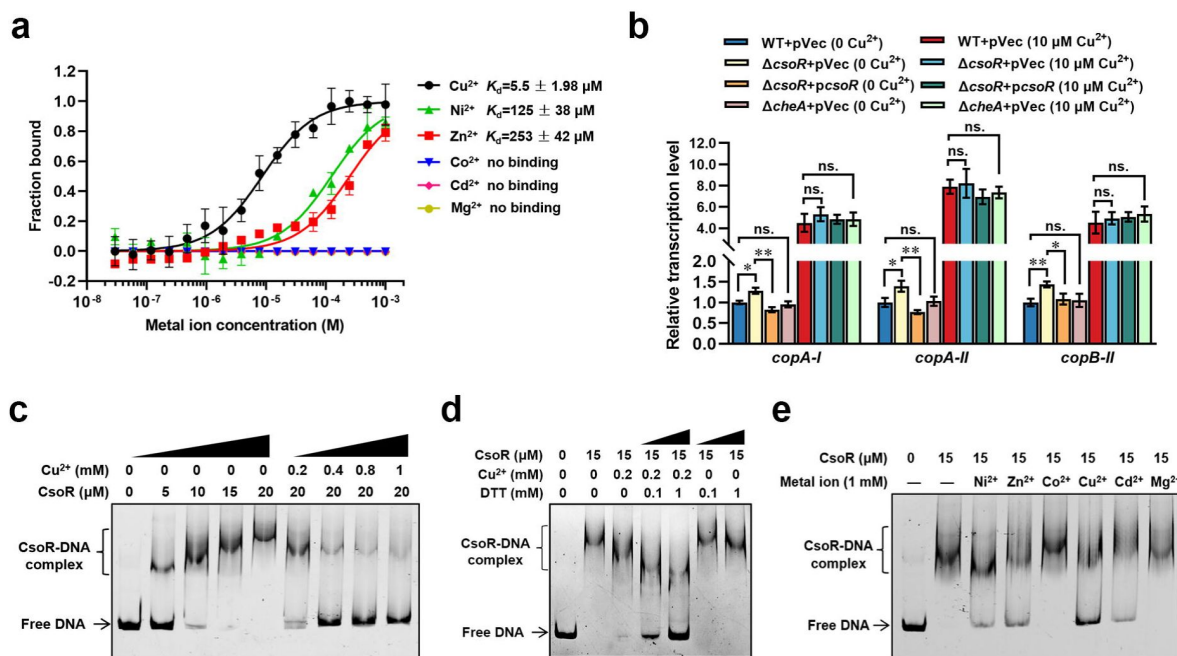


Fig. 4

CsoR is a metal-binding repressor.

(a) MST analysis of the interaction between CsoR-GFP and metal ions. CsoR-GFP (250 nM) was incubated with increasing concentrations of metal ions. (b) Analysis of relative transcription level of target genes in wild-type (WT+pVec), *csoR* mutant (ΔcsoR +pVec), complemented strain (ΔcsoR +pVec), and *cheA* mutant (ΔcheA +pVec) in the presence and absence of CuCl_2 (10 μM) by qRT-PCR. The results are the average of three independent assays. Error bars represent standard deviations. The asterisks represent statistically significant differences between the two compared strains (* $P < 0.05$, ** $P < 0.01$). "ns." represents none statistically significant between two compared strains. (c) Analysis for interactions between CsoR and *copA-I* promoter DNA using EMSA. (d) The effect of DTT/ CuCl_2 +DTT on the interaction between CsoR and *copA-I* promoter DNA. (e) The effect of indicated metal ions on the interaction between CsoR and *copA-I* promoter DNA. The concentrations of CsoR, metal ions, and DTT in panels c, d, and e added in each lane are shown above the gel. Free DNA and CsoR-DNA complex are indicated.

Copper inhibits the interaction between CheA and CsoR

Since CsoR interacted with CheA and bound copper, we wondered whether copper affected the interaction between CheA and CsoR. Thus, we investigated the interaction between CheA and CsoR under different CuCl_2 concentrations using MST. As revealed in **Fig. 5a**, CheA showed strong interaction with CsoR in the absence of Cu^{2+} ($K_d = 0.17 \pm 0.1 \mu\text{M}$), and the addition of Cu^{2+} (20 and 200 μM) led to increased binding constant ($0.59 \pm 0.2 \mu\text{M}$ and $2.15 \pm 0.97 \mu\text{M}$), indicating that copper decreased the interaction between CheA and CsoR. A similar trend was observed in the pull-down assay, in which the amount of CsoR bound by CheA gradually decreased with the concentration of Cu^{2+} increased from 2 to 20 μM (**Fig. 5b**).

A previous study on *M. tuberculosis* CsoR revealed three residues that played a vital role in copper-binding³⁹. The alignment assay showed that the three residues were conserved among CsoR homologs from several bacterial species, including the *P. putida* CsoR (**Fig. S7a**). To test the role of the three residues in copper-binding, we individually replaced each of the three residues (Cys40, His65, and Cys69) with alanine and tested the copper-binding ability of these mutated CsoRs (CsoR_{C40A}, CsoR_{H65A}, and CsoR_{C69A}). MST results showed that the CsoR_{C69A} displayed significantly decreased copper-binding ability compared to wild-type CsoR. In contrast, CsoR_{C40A} and CsoR_{H65A} showed a slight decrease in copper-binding ability compared with that of the wild-type CsoR (**Fig. S7b**), suggesting that the Cys69 residue of CsoR played a critical positive role in copper-binding. Besides, the results from EMSA also supported this conclusion, in which the mutation of C69A, but not C40A/H65A, noticeably inhibited the effect of Cu^{2+} on CsoR-DNA interaction (**Fig. S7c** and **S7d**). BTH assay revealed that the three point-mutated CsoRs interacted with CheA with a similar intensity to the wild-type CsoR (**Fig. S7e**). Results from the pull-down assay showed that the addition of Cu^{2+} significantly decreased the amount of CsoR_{C40A} and CsoR_{H65A} bound by CheA, but had less effect on the amount of CsoR_{C69A} bound by CheA under the same condition (**Fig. 5b**). Together, these results revealed that copper bound CsoR and inhibited its interaction with CheA.

Copper relieves the inhibition of CsoR on bacterial chemotaxis

Since CsoR interacted with CheA and inhibited its autophosphorylation, and copper hindered the interaction between CheA and CsoR, we speculated that copper might relieve the inhibition of CsoR on CheA autophosphorylation. To test this hypothesis, we added CsoR and copper to the reaction mixture containing CheA and then analyzed the autophosphorylation of CheA using [³²P]ATP[γP]. CsoR alone significantly inhibited CheA autophosphorylation, while adding CsoR and copper showed weaker inhibition on CheA autophosphorylation (**Fig. 5c**). Besides, CsoR_{C69A} showed similar inhibitory effects on CheA autophosphorylation in the presence and absence of Cu^{2+} (**Fig. 5c**), indicating that binding with CsoR was the prerequisite for Cu^{2+} to relieve the inhibition of CsoR on CheA autophosphorylation.

We further tested the effect of CsoR on chemotaxis in the presence of copper using the semisolid plate. As shown in **Fig. 5d**, in the absence of Cu^{2+} , the overexpression of point-mutated CsoR (CsoR_{C40A}/CsoR_{H65A}/CsoR_{C69A}) led to a similar decrease (about 40%) in chemotaxis as the overexpression of wild-type CsoR. In comparison, in the presence of 200 μM Cu^{2+} , the inhibitory effect of CsoR/CsoR_{C40A}/CsoR_{H65A} overexpression on chemotaxis was weaker (about 20%). However, the inhibitory effect of CsoR_{C69A} overexpression on chemotaxis was not affected by Cu^{2+} (**Fig. 5d**). These results demonstrated that binding copper relieved the inhibition of CsoR on bacterial chemotaxis.

CsoR inhibits chemorepellent response to copper

Excess copper is toxic to cells, and bacteria avoid high copper concentrations through chemotaxis. Since CsoR interacted with CheA and inhibited chemotaxis, we wondered about the role of CsoR in bacterial chemotaxis to copper. Thus, we tested the chemotaxis response of *P. putida* to copper

gradient and the role of CsoR and CheA in this response using semisolid plates. The copper gradient was achieved by placing an agar plug containing 200 mM CuCl₂ in the center of a semisolid plate, and bacterial cells were spotted two centimeters away from the plug to test their chemotaxis to Cu²⁺. An agar plug without CuCl₂ was also placed in the center of a semisolid plate and used as a negative control. As shown in **Fig. 6a**, the swimming zone of all tested strains was a circle shape in the control plate without CuCl₂. However, all swimming zones showed an oval shape in the plate with CuCl₂ gradient (**Fig. 6a**), in which the bacterial movement distance near the plug (D1) was short, and the movement distance from the plug (D2) was long, indicating that the strains showed chemorepellent response to CuCl₂. The RI value (Response index value, RI=D1/(D1+D2)) was further calculated to characterize the strength of the chemorepellent response to CuCl₂. The results showed that WT+pcsoR displayed a higher RI value (0.428 ± 0.015) than WT+pVec (0.373 ± 0.021) (**Fig. 6b**). Meanwhile, ΔcsoR+pVec showed a lower RI value (0.324 ± 0.013) than WT+pVec, and complementation increased the RI value to wild-type level (**Fig. 6b**). These results suggested that CsoR inhibited the chemorepellent response to copper. Besides, both cheA deletion mutant (ΔcheA+pVec) and cheA csoR double deletion mutant (ΔcsoRΔcheA+pVec) displayed no chemotaxis ability in either the presence or absence of copper gradient (**Fig. 6a** and **6b**), indicating that CsoR inhibited chemotaxis ability in a CheA-dependent manner.

Using time-lapse microscopy experiments and cell-tracking analysis, we further examined the bacterial chemorepellent response to Cu²⁺. In the control group without chemokine (Cu²⁺ gradient), cells of all tested strains swam randomly in all directions (**Fig. 6c**). The center of mass (defined as the average of all single cell endpoints, and it reflects the movements of target strain and the strength of chemotaxis response) of all tested strains showed no apparent difference (**Fig. 6d**). In the group with chemokine (Cu²⁺ gradient), cells of WT+pVec, WT+pcsoR, ΔcsoR+pVec, and ΔcsoR+pcsoR migrated towards the lower concentration of Cu²⁺ (**Fig. 6c**). In comparison, cells of ΔcheA+pVec and ΔcsoRΔcheA+pVec still swam randomly in all directions (**Fig. 6c**), indicating that *P. putida* cells showed a chemorepellent response to Cu²⁺ in a CheA-dependent manner. The center of mass value of WT+pcsoR (13.85 ± 1.92 μm) was smaller than that of WT+pVec (21.77 ± 3.60 μm) (**Fig. 6d**). Meanwhile, the center of mass value of ΔcsoR+pcsoR (14.16 ± 1.41 μm) was smaller than that of ΔcsoR+pVec (21.39 ± 2.02 μm) (**Fig. 6d**), suggesting that CsoR inhibited bacterial chemorepellent response to Cu²⁺. The velocities (cell migration speeds) of cells from WT+pcsoR, ΔcsoR+pVec, and ΔcsoR+pcsoR were similar to that from WT+pVec both in the presence and absence of Cu²⁺ gradient, implying that CsoR had no evident influence on bacterial migration speed. In contrast, the velocities of cells from ΔcheA+pVec and ΔcsoRΔcheA+pVec were lower than that from WT+pVec (**Fig. 6e**), indicating that CheA played a positive role in bacterial migration speed. Together, these results demonstrated that CsoR inhibited the chemorepellent response to copper in a CheA-dependent manner.

The interaction between CheA and CsoR exists in several bacterial species

The role of CsoR in regulating copper resistance has been reported in several bacterial species, including *Acidithiobacillus caldus*⁴², *Bacillus subtilis*⁴³, *Bradyrhizobium diazoefficiens*⁴⁴, *Corynebacterium glutamicum*⁴⁵, *Listeria monocytogenes*⁴⁶, *M. tuberculosis*⁴⁰, *Staphylococcus aureus*⁴⁷, *Streptomyces lividans*⁴⁸, and *Thermus thermophilus*⁴⁹. BLAST results showed that four of the nine above species (*A. caldus*, *B. diazoefficiens*, *B. subtilis*, and *L. monocytogenes*) had both cheA and csoR on their genomes. Besides, in addition to *P. putida*, cheA and csoR coexist in other *Pseudomonas* species, including *Pseudomonas fluorescens*, *Pseudomonas syringae*, and *Pseudomonas stutzeri*. We wondered whether the CheA-CsoR interaction also occurred between proteins from these strains. Thus, we tested the interaction between CheA and CsoR of the same strain via BTH assay. The result showed that the CheA-CsoR interaction existed between proteins from *A. caldus*, *B. subtilis*, *P. syringae*, and *P. stutzeri* (**Fig. 7**). However, CheA and CsoR from *B. diazoefficiens*, *L. monocytogenes*, and *P. fluorescens* showed no apparent interaction (**Fig. 7**). Besides, the intensity of CheA-CsoR interaction was more vigorous between the two proteins from

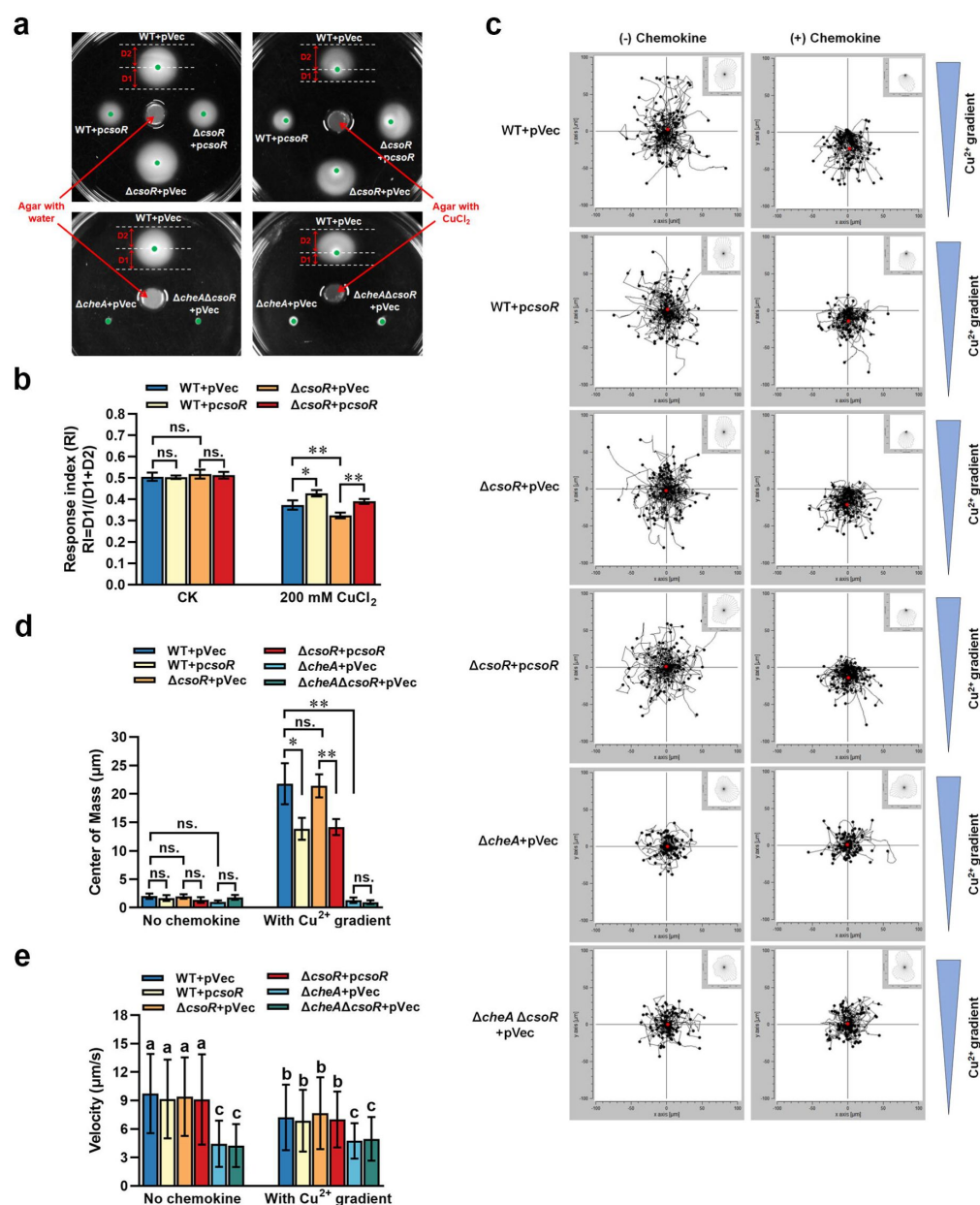


Fig. 6

Role of CsoR in bacterial chemotaxis to copper.

(a) Chemotaxis of indicated strains in the absence and presence of copper gradient. The chemotaxis rings and chemotaxis distance (D1/D2) were indicated by arrows. The red arrows pointed at the agar plug with or without copper in the center of the plate. The green dots represented the sites where the bacteria were initially inoculated on the semisolid plate. The assay was performed with three repeats, and a representative photo was shown. (b) RI value (D1/(D1+D2)) of indicated strains shown in panel a. (c) Aggregated trajectories of individual tested strain cells in the absence and presence of copper gradient. The tracking data presented is a composite of two experiments performed in duplicate (n = 100 cells). The overall directionality of migration is depicted in the rose diagram in the upper right corner of each single-track summary. (d) Center of mass of tested strains in the presence of copper gradients. It represents the average of all single-cell endpoints. The results of panels b and d are the average of three independent assays. Error bars represent standard deviations. The asterisks represent statistically significant differences between the two indicated strains (**P* < 0.05, ***P* < 0.01). "ns." represents no statistically significant between the two indicated strains. (e) Velocity analysis of indicated strains in the presence or absence of copper gradient (n = 100 cells). The lowercase letters above each bar in panel e indicate significant differences (*P* < 0.05).

B. subtilis, but weaker between that from *A. caldus*, *P. syringae*, and *P. stutzeri* (Fig. 7). These results suggested that except in *P. putida*, the interaction between CheA and CsoR also existed in other bacterial species.

Discussion

Integrating components from different systems provides a straightforward mechanism for coordinating signaling from various systems. This study identified an interaction between the chemotaxis kinase CheA and the copper-responsive transcriptional repressor CsoR in *P. putida*. Further analysis revealed that CsoR inhibited bacterial chemotaxis via interacting with CheA and hindering its autophosphorylation. Meanwhile, CsoR regulated copper resistance by modulating the expression of copper-resistance genes. Together with previous reports^{50–52}, we proposed a potential model to describe the function of CsoR in regulating copper resistance and bacterial chemotaxis. As shown in Fig. 8, under low copper levels, CsoR molecules exist mainly in none copper-binding status (free CsoR) in the cell, and the free CsoR forms tetramer and binds to promoters of copper-resistance genes (such as *copA-I*), leading to repressed gene transcription and copper resistance ability. Meanwhile, the free CsoR interacts with CheA and inhibits its autophosphorylation, decreasing bacterial chemotaxis ability. Under high copper levels, more copper-binding CsoR molecules exist, and copper-binding changes the conformation of the CsoR tetramer and releases CsoR from promoters, leading to increased gene transcription and copper resistance. Besides, the copper-binding of CsoR decreases the interaction between CsoR and CheA, which relieves the inhibition of CsoR on CheA autophosphorylation, resulting in increased chemotaxis ability.

In classical chemotaxis signaling, MCP on the cell membrane senses external signaling molecules and regulates bacterial chemotaxis by mediating CheA autophosphorylation activity^{5,7}. MCPs can directly bind diverse external signaling molecules, such as amino acids, dipeptides, sugars, tricarboxylic acid cycle intermediates, aromatic molecules, and inorganic phosphate⁵³. However, there are only a few reports on the relationship between MCP and metal ions^{54–56}, and no evidence supports that MCP senses metal ions by direct binding. It is possible that bacteria sense and trigger chemotaxis to metal ions differently from that they sense and trigger chemotaxis to external signaling molecules like amino acids. Our results provide a mechanism by which bacteria sense copper and regulate chemotaxis via the copper-responsive repressor CsoR. Through the interaction between CsoR and CheA, bacteria coordinately regulated chemotaxis and resistance to copper stress, which would favor the bacteria to better adapt to complex environments. Besides, the interaction between CsoR and CheA was not limited to the proteins from *P. putida*, and it was also found in proteins from several other bacterial species (Fig. 7), implying that the regulation of chemotaxis and resistance to copper via the interaction between CsoR and CheA might be a widespread regulatory mechanism.

Although the *P. putida* CsoR functioned as a copper-responsive regulator to modulate the expression of copper-resistance genes, its effect on gene expression was much weaker than its homologous protein in other bacterial species. In *M. tuberculosis*, *B. subtilis*, *C. glutamicum*, *L. monocytogenes*, and *S. aureus*, deletion of *csoR* resulted in an about 10-fold increase in the expression of target genes in the absence of copper^{40,43,45,46,47}. In contrast, deletion of *csoR* in *P. putida* led to a slight but reproducible increase (about 1.3-fold) in gene expression in the absence of copper (Fig. 4b). This difference might be attributed to the existence of several key regulators that activated the expression of copper-resistance genes in response to copper in *P. putida*, such as CueR and CopR. CueR positively regulated the expression of *cueA*, encoding a copper-transporting P1-type ATPase that played a crucial role in copper resistance²⁸. CopR was essential for expressing several genes implicated in cytoplasmic copper homeostasis, such as *copA-II*, *copB-II*, and *cusA*^{30,57}. The existence of these positive regulators makes the function of CsoR

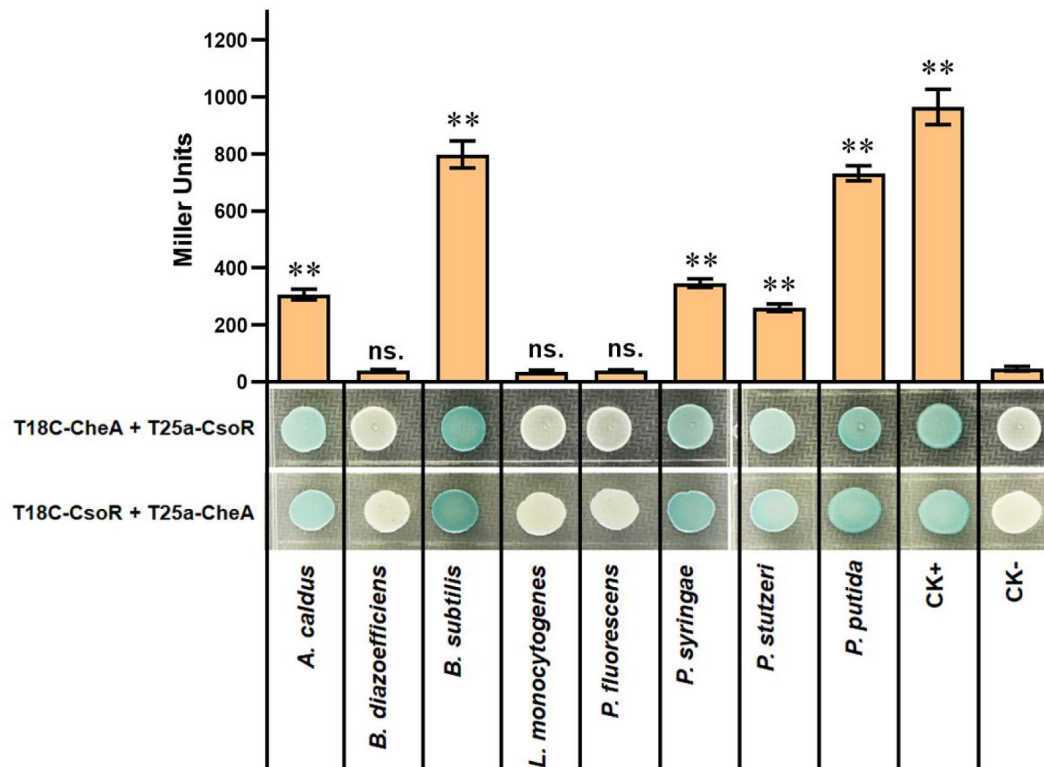


Fig. 7

The interaction between CheA and CsoR from indicated bacterial species.

The interaction between CsoR and CheA was tested by using BTH. The LacZ activities of colonies were shown above the colonies. The results are the average of three independent assays. Error bars represent standard deviations. The asterisks above the column denote significant differences (** $P < 0.01$) between indicated strains and CK-strain analyzed by Student's t-test. "ns." represents none statistically significant between indicated strain and CK-strain.

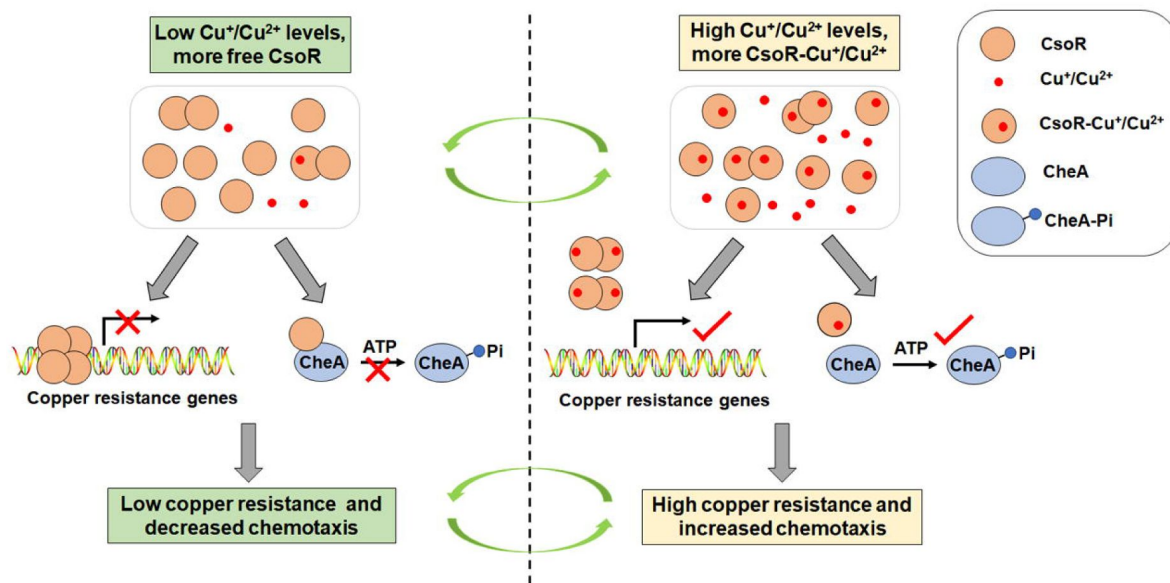


Fig. 8

A proposed model for describing how CsoR coordinates chemotaxis and resistance to copper in *P. putida*.

Under low $\text{Cu}^+/\text{Cu}^{2+}$ levels, more non- $\text{Cu}^+/\text{Cu}^{2+}$ -binding CsoR molecules (free CsoR) exist in the cell, and the free CsoR forms tetramer and binds to promoters of copper-resistance genes (such as *copA-I* and *copA-II*), leading to repressed gene transcription and low copper resistance. Meanwhile, free CsoR interacts with CheA and inhibits its autophosphorylation activity, decreasing chemotaxis ability. In contrast, more $\text{Cu}^+/\text{Cu}^{2+}$ -binding CsoR molecules exist under high $\text{Cu}^+/\text{Cu}^{2+}$ levels, and the $\text{Cu}^+/\text{Cu}^{2+}$ -binding changes the conformation of the CsoR tetramer and releases CsoR from target promoters, leading to increased gene transcription and copper resistance. Besides, $\text{Cu}^+/\text{Cu}^{2+}$ -binding of CsoR decreases the interaction between CsoR and CheA, which relieves the inhibition of CsoR on CheA autophosphorylation, resulting in increased chemotaxis ability.

a secondary or even dispensable insurance in the expression of copper-resistance genes. Consistent with this, there is no CosR homolog in *P. aeruginosa*, and copper homeostasis in *P. aeruginosa* is mainly controlled by CueR and CopR^{29,30}.

Through pull-down, BTH, and BiFC assays, we obtained 16 new CheA-interacting proteins involved in different physiological processes (Table S1). Among the 16 proteins, five proteins (CsoR, IspG, NfuA, PhaD, and PP_1644) inhibited bacterial chemotaxis on semisolid plates (**Fig. 2a**). Our study here focused on the physiological role of CsoR-CheA interaction. Still, the function of other interactions remained unclear. PhaD is a TetR family transcriptional regulator that behaves as a carbon source-dependent activator of the *pha* cluster related to polyhydroxyalkanoates (PHAs) biosynthesis^{58,59}. Bacterial PHAs are isotactic polymers synthesized under unfavorable growth conditions in the presence of excess carbon sources. PHAs are critical in central metabolism, acting as dynamic carbon reservoirs and reducing equivalents⁶⁰. The interaction between PhaD and CheA leads one to speculate that there might be some connection between PHA synthesis and bacterial chemotaxis. Another CheA-interacting protein, PP_1644, also attracts our interest. PP_1644 is annotated as a NAD(P)H dehydrogenase involved in cyclic electron transport and respiration processes. Exploring the physiological role of the interaction between CheA and these proteins in the future helps to reveal the association between the chemotaxis process and other physiological metabolisms.

Materials and Methods

Bacterial strains and growth conditions

All strains and plasmids used in this study are listed in Supplementary Table S2. Unless specifically mentioned, *E. coli* strains were grown in lysogeny broth (LB) medium at 37°C. *P. putida* KT2440 and its derivative strains were cultured at 28°C in LB medium or chemically defined M9 minimal medium supplemented with 40 mM glucose as carbon source. Antibiotics were used, when required for plasmid maintenance or transformants screening, at the following concentrations: kanamycin (50 mg/L), carbenicillin (50 mg/L), chloramphenicol (25 mg/L), and gentamycin (20 mg/L for *E. coli* or 40 mg/L for *P. putida*).

Plasmid and strain construction

The routine cloning of DNA fragments into plasmid was performed by following a T5 exonuclease-dependent method⁶¹. Briefly, a 14 base pairs (bp) homologous end was added to the 5' of primer during synthesizing. Amplified DNA fragments and linearized plasmid containing the same homologous end were incubated in a reaction buffer containing T5 exonuclease (0.04 U) and left at 30 for 40 min before transforming into *E. coli* competent cells. Primers used for plasmid construction are listed in Supplementary Table S3. All cloning steps involving PCR were verified by commercial sequencing (Tsingke, Wuhan, China).

Gene deletion mutant was constructed by homologous recombination using the suicide plasmid pBBR401. For example, to construct a markerless *P. putida* *csoR* deletion mutant, ~800 bp from the chromosomal regions flanking *csoR* (upstream region and downstream region) were PCR-amplified. The PCR products were cloned into pBBR401 to create pBBR401-*csoR*UP-DOWN. Then, the final plasmid was transferred to *P. putida* by electroporation. The integration strain was selected on plates containing gentamicin. After subculturing the integration strain in LB medium without antibiotics six times (12 h each time), single colonies were obtained by plate streaking. Then, colonies losing gentamicin resistance were kept for further verification. The *csoR* delete mutant was confirmed by PCR and sequencing.

To generate a *csoR/cheA* overexpression plasmid, a DNA fragment containing the complete *csoR/cheA* was PCR amplified. The product was cloned into expressional vector pBBR403 to yield pBBR403-*csoR/cheA*. The expression of *csoR/cheA* on pBBR403-*csoR/cheA* was controlled by an inducible *tac* promoter. To construct a vector for target protein expression and purification, we amplified and cloned the target gene into pET-28a with 6 × His tag or pHS-Strep with Strep II tag. Overlapping PCR was used to create point mutations of CsoR. To construct a C40A point mutation in CsoR, we amplified two fragments with two primer pairs (CsoR_{C40A} s1/CsoR_{C40A} a1 and CsoR_{C40A} s2/CsoR_{C40A} a2). The CsoR_{C40A} a1 and the CsoR_{C40A} s2 shared reverse complementary sequences containing the point mutation in which the original TGC codon of arginine was replaced by GCC of alanine. The two fragments were mixed in a 1:1 ratio to perform overlapping extension. The final PCR product was cloned into pET-28a and pBBR403. The mutation in *csoR* was confirmed by sequencing.

Expression and purification of His/Strep II-tagged proteins

For the expression of His/Strep II-tagged protein, overnight culture of *E. coli* BL21 carrying the construct of target proteins was 1:100 diluted into LB medium and incubated for 4 h at 37°C. Then, 0.4 mM IPTG (isopropyl-D-thiogalactopyranoside) was added to induce protein expression. After 4 h incubation at 16°C, cells were harvested and resuspended in lysing buffer (10 mM Tris-Cl [pH 7.8], 300 mM KCl, and 10% (w/v) glycerol). The harvested cells were lysed using a pressure cell breaking apparatus, and cell debris was removed by centrifugation at 15000 rpm for 20 min. The supernatants were then filtered through a 0.22-μm-pore-size filter and loaded onto a Ni-NTA Resin column (for His-tagged protein) or Strep-Tactin Resin column (for Strep II-tagged protein). Target proteins were eluted using an imidazole gradient (50/100/150/250mM imidazole for His-tagged protein) or 5 mM biotin (for Strep II-tagged protein) and then dialyzed overnight against lysing buffer to remove imidazole. The concentrations of obtained proteins were determined using BCA assay.

Protein-protein pull-down assay

Protein-protein pull-down assay was used to identify CheA-interacting protein and test the effect of copper on CheA-CsoR interaction. Briefly, 6 × His/Strep II-tagged CheA was induced and loaded to a Ni-NTA/Strep-Tactin column as described above. Then, overnight cultured wild-type KT2440/BL21 strain expressing CsoR was harvested, lysed, and filtered before adding to the same column. For the pull-down assay to identify CheA-interacting protein, the same volume of wild-type extract was added to a blank Ni-NTA column as a negative control. For the pull-down assay to test the effect of copper on CheA-CsoR interaction, various amounts of CuCl₂ were mixed with the cell extract containing CsoR before being added to the Strep-Tactin column. Then, the columns were sealed and incubated at 4°C with 40 rpm shaking. After 2 h incubation, the supernatant was removed, and the columns were washed with lysing buffer containing 20 mM imidazole (for Ni-NTA column) or lysing buffer (for Strep-Tactin column) to wash the unspecific binding protein away. Then, elution buffer containing 250 mM imidazole (for the Ni-NTA column) or 5 mM biotin (for the Strep-Tactin column) was added to wash down all proteins on the column. The eluted proteins were collected and resolved by 12.5% SDS-PAGE followed by Coomassie blue staining and mass spectrometry analyses.

Mass spectrometry (MS)-based protein sequencing

After Coomassie blue staining of eluted proteins obtained from pull-down assay, the whole lane of the experimental or control sample was excised from gels and prepared for MS analysis. Protein from the excised gels was extracted with a Micro Protein PAGE Recovery Kit (Sangon Biotech, China) following the operating instructions. Then, trypsin digestion of extracted protein was performed with 1 g trypsin (Promega, USA) and incubated at 37°C overnight. The digestion was terminated by adding trifluoroacetic acid (TFA). Then, desalting was subsequently performed

using Zip-tip (Merck Millipore, Ireland). Peptides were eluted from the Zip-tip with 50 μ L of matrix solution (5 mg/mL α -cyano-4-hydroxycinnamic acid, 50% acetonitrile, 0.1% TFA). The supernatant was collected and concentrated to a final volume of 10 μ L in a centrifugal concentrator.

The samples were analyzed using the MALDI-TOF/TOF mass spectrometer (Applied Biosystems, USA). Mass spectra were recorded in the positive-ion mode, averaging 2500 laser shots per spectrum. Mass spectra (excluding trypsin autolytic peptides and other known background ions) were searched against the *P. putida* proteome from the UniProt database to identify the proteins. The search was performed using trypsin digestion, allowing two missed cleavages, specifying carbamidomethyl-Cys as a fixed modification, and setting a peptide mass tolerance of ± 1.6 Da. The global false discovery rate (FDR) cutoff for peptide and protein identification was set to 0.01. An intensity-based absolute quantification (iBAQ) algorithm was used to rank the relative abundance of different proteins as previously described⁶². iBAQ percentage of specific proteins in the experimental sample (iBAQ_T (%)) and control sample (iBAQ_CK (%)) were used to represent relative protein concentration. $\log_2$ (iBAQ_T/iBAQ_CK) fold change of ≥ 2 or ≤ -2 and a *P* value of ≤ 0.05 was considered significantly different.

Bacterial two-hybrid (BTH) assay

For bacterial two-hybrid analysis of protein-protein interactions of *P. putida* proteins expressed in *E. coli*, each ORF was cloned in-frame with the T18 and T25 fragments of adenylate cyclase ORF in vectors pUT18C and pKT25. Primers used to amplify each ORF are listed in Table S3. The resulting vectors were co-transformed into *E. coli* BTH101 and plated onto LB agar plate supplemented with 50 mg/L carbenicillin, 50 mg/L kanamycin, 40 mg/L 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal), and 0.5 mM IPTG, and incubated at 28°C for 48 h. Three co-transformants for each assay were cultured to stationary phase in LB broth at 28°C, then spotted onto an LB agar plate supplemented as above, and incubated for 60 h at 28°C. Plates were then photoed on a Tanon 2500 scanner. After the photographs were taken, the colonies on the plates were scraped off, and the LacZ activities of obtained cells were measured using o-nitrophenyl- β -galactopyranoside (ONPG) as substrate, as described before⁶³. The experiments were repeated three times with three technical repeats per sample, and the data are presented as Miller units.

Bimolecular fluorescence complementation (BiFC) assay

BiFC was used to analyze protein-protein interactions as previously described⁶⁴. Briefly, to determine the interaction between two interested proteins (such as CheA and CsoR), CheA was fused to KN151 (the N-terminal of mLumin), and CsoR was fused to LC151 (the C-terminal of mLumin), yielding a recombinant plasmid pBBR403-CheA-KN151-CsoR-LC151. Then, the recombinant plasmid was transformed into the wild-type *P. putida* strain. Transformants were picked and cultured in LB medium containing 40 mg/L gentamycin and 0.5 mM IPTG for 24 h at 28°C. Then, images of the dark-field and bright field of the transformant cells were obtained using FV1000 CLSM (Olympus, Japan) equipped with a 100 $\times$ /1.4 oil immersion objective lens. Besides, the transformant cells were washed twice with 0.9% NaCl, and then fluorescence intensities and OD₆₀₀ were measured using a Spark microplate reader (Tecan, Switzerland). Fluorescence intensity and OD₆₀₀ were detected using the black and transparent microplate, respectively. The excitation and the emission wavelength to detect fluorescence were 587 nm and 620 nm, respectively, and the experiment was repeated twice with triplicates.

Microscale thermophoresis (MST) assay

MST was performed to analyze the interaction between two proteins or proteins and metal ions. Briefly, to test the interaction between CsoR and copper. A green fluorescent protein (GFP) encoding gene was fused to the end of *csoR* in pET-28a-*csoR* to achieve fusion expression. The fusion protein CsoR-GFP was induced and purified as described above in protein induction and purification. The obtained CsoR-GFP was dialyzed with MST buffer (50 mM Tris-HCl [pH 7.8], 150

mM NaCl, 0.05% Tween 20). The MST assay was performed on a Monolith Instrument NT.115 device using standard treated capillaries (NanoTemper Technologies, Germany). The concentration of CsoR-GFP was constant at 250 nM, and the CuCl_2 concentration was varied from 0.031 to 1000 μM with a 2-fold gradient. The experiment was recorded using the Nano-BLUE fluorescent detector. Measurements were performed in MST buffer. The MO. Affinity Analysis software (version 2.3) was used to calculate the dissociation constant (K_d) from triplicate reads of measurements.

***In vitro* phosphorylation assays**

For the autophosphorylation reaction, purified CheA (3 μM) was incubated in phosphorylation buffer containing 50 mM Tris-HCl [pH 7.5], 15 mM MgCl_2 , and 50 mM NaCl. The reaction was initiated by adding 0.03 μCi of [^{32}P]ATP[γP] (PerkinElmer, USA) to the mixture. SDS-PAGE loading buffer containing SDS and EDTA was added to the mixture to terminate the reaction at the indicated time. To test the effect of target proteins on CheA autophosphorylation, target protein (10 μM) was mixed with CheA (3 μM) for 10 min before adding [^{32}P]ATP[γP]. To test the transphosphorylation reaction, CheA was autophosphorylated before CheY/target protein (10 μM) was added to the mixture, and the reaction mixture was incubated at 30°C for different time intervals before being terminated with SDS-PAGE loading buffer. Samples were heated at 95°C for 5 min and then resolved by 12.5% SDS-PAGE. After drying of the gels, products were visualized by autoradiography.

RNA extraction and real-time RT-PCR (qRT-PCR) assay

P. putida cells were cultured in M9 minimal medium supplemented with 40 mM glucose as carbon source for 24 h. Then, cells were harvested and washed thrice with sterilized phosphate buffer saline (PBS) before being divided into two equal parts. One part was resolved with fresh M9 medium and another with M9 medium containing 10 μM CuCl_2 . After 30 min incubation, cells were harvested for RNA extraction using a total RNA extraction reagent (Vazyme R401-01, China) as recommended by the manufacturer. 1 μg extracted RNA was digested with DNase I and reverse transcribed to cDNA using a reverse transcription kit (Takara RR047A, Japan), and cDNA was used as the template for qRT-PCR analysis. The qRT-PCR assay was performed using Power SYBR™ Green PCR mix (Applied Biosystems 4367659, USA) and analyzed using a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, USA). The *rpoD* gene was selected as an internal control. The primers used in qRT-PCR analysis are listed in Supplementary Table S3. All experiments were performed thrice with three technical repeats per sample.

Electrophoretic mobility shift assay (EMSA)

EMSA was used to test the interaction between CsoR and *copA-I* promoter DNA. Equal amounts of DNA (60 ng) were added to binding reactions with various quantities of CsoR in binding buffer (10 mM Tris-Cl [pH 7.8], 50 mM KCl, 20 mM MgCl_2 , 5% glycerol, 20 μL total reaction volume). CsoR was incubated with promoter DNA for 20 min at room temperature. Reaction mixtures containing $\text{Cu}^{2+}/\text{DTT}/\text{Cu}^{2+}+\text{DTT}$ were performed as described above, except $\text{Cu}^{2+}/\text{DTT}/\text{Cu}^{2+}+\text{DTT}$ was incubated with CsoR for 10 min before adding DNA. All reaction solutions were loaded onto 5% acrylamide gel and electrophoresed at 150 V for 40 min in $0.5 \times \text{TBE}$ buffer (45 mM Tris-Cl [pH 7.8], 45 mM borate, 1 mM EDTA). Gels were stained with ethidium bromide before being digitized using a scanner (Tanon 2500, China).

Bacterial chemotaxis assay

The chemotaxis ability of *P. putida* strains was assessed by using semisolid plate and μ -slide Chemotaxis plate (Ibidi 80326, Germany). For the method with semisolid plates, an agar plug (1% agar) containing 200 mM CuCl_2 was placed in the center of a LB semisolid plate (0.25% agar) and left at room temperature for 12 h to achieve a CuCl_2 gradient on the semisolid plate. An agar plug without CuCl_2 was also placed in the center of a semisolid plate and used to test bacterial

chemotaxis without copper. For the assay to investigate the effect of copper on bacterial chemotaxis, 200 μM CuCl_2 (final concentration) was mixed with semisolid LB before making a semisolid plate. Overnight growth *P. putida* cells were washed and resuspended with fresh M9 medium and adjusted to the same optical density ($\text{OD}_{600} = 0.5$). Then, 2 μL of resuspended cultures was spotted 2 centimeters away from the agar plug/plate center, and the plate was incubated for 16 h (for the control plate) or 18 h (for the copper-containing plate) at 28 °C before digital photographs were taken. The distance from the point of inoculation to the edge of the colony growth closest to the agar plug (D1) and the distance from the point of inoculation to the edge of the colony growth farthest from the agar plug (D2) were measured. The response index (RI) value was calculated to characterize the bacterial response to CuCl_2 as previously described³⁶. The RI was calculated using the following equation: $\text{RI} = \text{D1}/(\text{D1} + \text{D2})$. RI values greater than 0.52 and less than 0.48 correspond to attractant and repellent responses, and intermediate values represent nonresponses.

For the method with μ -slide Chemotaxis plate (a commercially available microfluidic device with a channel connecting two reservoirs), the reservoirs were filled with the two bacterial solutions (with or without CuCl_2) following a modified version of the manufacturer's "Fast Method" protocol. Briefly, overnight cultures were inoculated with M9 medium and grown at 28 °C, 180 rpm, until they reached an optical density (OD_{600}) between 0.3 and 0.35. 1 ml of bacterial culture was washed twice with fresh M9 medium by centrifugation (3 min at 3000 rpm), and finally diluted to a target OD_{600} of 0.015 with fresh M9 supplemented with 0.01% Tween 20, with or without 2 mM CuCl_2 for injection into the chemotaxis device. First, the entire device was overfilled with buffer free of CuCl_2 or bacteria through the filling ports, and then the central channel's ports were closed with plugs. About 65 μL was removed from one reservoir, replaced by 65 μL of CuCl_2 -free bacterial solution, and then this reservoir's ports were closed. Finally, all liquid was removed from the other reservoir and replaced with a bacterial solution containing CuCl_2 . For control measurements, neither bacterial solution contained CuCl_2 . Phase contrast microscopy recordings were obtained at room temperature on a Nikon Ti-E inverted microscope using an sCMOS camera (PCO Edge 4.2) and a 40 x objective lens. Recordings were obtained starting from 20 min after filling the device. Three 15 s-long recordings of cells in the observation area were obtained at 15 fps. Then, single-cell tracking analysis was performed using ImageJ's Manual Tracking and Chemotaxis Tool plugins.

Statistical analysis

Statistical analyses were performed using Graph-Pad Prism (version 9.0.0). Student's t-test or an analysis of variance (ANOVA) was used to analyze the significance of differences in LacZ activity, fluorescence intensity, swimming zone diameter, gene expression, response index, center of mass, and velocity. A *P* value less than 0.05 was considered statistically significant.

Data availability

Relevant data supporting the critical findings of this study are available within the article and the Supplementary Information file. The absorbance, microscopic photographs, gels, plates, and EMSA data generated in this study have been provided in the Source data file.

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Additional files

Supplementary information [↗](#)

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Reviewer #2 (Public review):

Summary:

This manuscript focuses on the apparent involvement of a proposed copper-responsive regulator in the chemotactic response of *Pseudomonas putida* to Cu(II), a chemorepellent. Broadly, this area is of interest because it could provide insight into how soil microbes mitigate metal stress. Additionally, copper has some historical agricultural use as an antimicrobial, thus can accumulate in soil. The manuscript bases its conclusions on an *in vitro* screen to identify interacting partners of CheA, an essential kinase in the *P. putida* chemotaxis-signaling pathway. Much of the subsequent analysis focuses on a regulator of the CsoR/RcnR family (PP_2969).

Weaknesses:

The data presented in this work does not support the model (Figure 8). In particular, PP_2969 is linked to Ni/Co resistance not Cu resistance. Further, it is not clear how the putative new interactions with CheA would be integrated into diverse responses to various chemoattract/repellents. These two comments are justified below.

PP_2969

- The authors present a sequence alignment (Figure S5) that is the sole based for their initial assignment of this ORF as a CsoR protein. There is conservation of the primary coordinating ligands (highlighted with asterisks) known to be involved in Cu(I) binding to CsoR (ref 31). There are some key differences, though, in residues immediately adjacent to the conserved

Cys (the preceding Ala, which is Tyr in the other sequences). The effect of these change may be significant in a physiological context.

- The gene immediately downstream of PP_2969 is homologous to *E. coli* RcnA, a demonstrated Ni/Co efflux protein, suggesting that P2969 may be Ni or Co responsive. Indeed PP_2970 has previously been reported as Ni/Co responsive (J. Bact 2009 doi:10.1128/JB.00465-09). The host cytosol plays a critical role in determining metal-response, in addition to the protein, which can explain the divergence from the metal response expected from the alignment.
- The previous JBact study also explains the lack of an effect (Figure 5b) of deleting PP_2969 on copper-efflux gene expression (copA-I, copA-II, and copB-II) as these are regulated by CueR not PP_2969 consistent with the previous report. Deletion of CsoR/RcnR family regulator will result in constitutive expression of the relevant efflux/detoxification gene, at a level generally equivalent to the de-repression observed in the presence of the signal.
- Further, CsoR proteins are Cu(I) responsive so measuring Cu(II) binding affinity is not physiologically relevant (Figures 5a and S5b). The affinities of demonstrated CsoR proteins are 10-18 M and these values are determined by competition assay. The MTS assay and resulting affinities are not physiologically relevant.
- The DNA-binding assays are carried out at protein concentrations well above physiological ranges (Figs 5c and d, and S5c, d). The weak binding will in part result from using DNA-sequences upstream of the copA genes and not from from PP_2970.

CheA interactions

There is no consideration given to the likely physiological relevance of the new interacting partners for CheA.

- How much CheA is present in the cell (copies) and how many copies of other proteins are present? How would specific responses involving individual interacting partners be possible with such a heterogenous pool of putative CheA-complexes in a cell. For PP_2969, the affinity reported (Figure 5A) may lay at the upper end of the CsoR concentration range (for example, CueR in Salmonella is present at ~40 nM).
- The two-hybrid system experiment uses a long growth time (60 h) before analysis. Even low LacZ activity levels will generate a blue colour, depending upon growth medium (see doi: 10.1016/0076-6879(91)04011-c). It is also not clear how Miller units can be accurately or precisely determined from a solid plate assay (the reference cited describes a protocol for liquid culture).

Comments on revised version:

The authors have replied in detail to the various comments about the original manuscripts. However, the responses are generally lengthy rationalisations of the original interpretation of the data and do not fundamentally address critical concerns raised about the physiological relevance of the results. The response appears to rest on the assumption that the numerous interacting partners obtained from the initial screen are all true positives and that all subsequent experimental results are interpreted to justify that assumption. In the case of CsoR, the experimental results and interpretation are inconsistent with previously published studies of the metal and DNA-binding properties of CsoR proteins. The following points reiterate comments from the previous review, in the hopes that the authors will, at the very least, consider the likelihood that the "CsoR" protein they have identified is in fact responsive to a different metal. Further, that the authors consider multiple possible interpretations of the data, particularly those that are inconsistent with the model/hypothesis (and take this into account in their experimental design).

- (Figure 4) Almost all purified proteins will bind Cu(II) most tightly in vitro, followed by Zn(II) and Ni(II). This behaviour is a consequence of the Irving-Williams affinity series (doi.org/10.1038/162746a0 and doi.org/10.1039/JR9530003192, especially Figure 4) and is not considered an indicator of physiological metal preference. Biomolecules will exhibit the same behaviour as small organic ligands towards first row transition ions because of the flexibility of their structures. Thus, the results obtained are unsurprising and, because of the method used, have no physiological relevance.
- The authors cite other in vivo work as evidence for varied metal-response by regulator proteins. However, experiments in these citations are of limited relevance because some focus on other structural classes of metalloregulator proteins (so not relevant here) while others focus on changes in metal accumulation by overexpression of the regulator protein, with no examination of the metal-specificity of the efflux protein (the key determinant of the physiological response of the regulator protein - why turn on expression of an efflux protein that can't pump out a particular metal? Finally, adding equivalent concentrations of metals to growing cells is not a good comparison as metals are toxic at different concentrations. The regulators will only have evolved to be just good enough, not perfect, with respect to selectivity. Laboratory experimental conditions often explore non-physiological conditions.
- It is also important to re-emphasise the authors' own statements on lines 90-93 that *P. putida* has a CueR protein. This is consistent with the phylogenetic distribution of CueR proteins in gram-negative bacteria. The CsoR proteins, in contrast, are found only in gram-positive bacteria. This inconsistency is ignored by the authors.
- The implications of the Irving-Williams series on metal-specific responses of bacterial metalloregulator proteins are described in the following references: 10.1016/j.cbpa.2021.102095, 10.1074/jbc.R114.588145, and 10.1038/s41589-018-0211-4). The last reference of this set provides an experimental basis for why metalloregulator affinities for Cu (and Zn and Ni) are so tight (and why the values obtained in Figure 4 in this manuscript are not relevant).
- Similarly, the previous experimental studies of CsoR proteins not cited by the authors (10.1021/ja908372b 10.1021/bi900115w) provide rigorous experimental approaches for measuring metal and DNA-binding affinities and further highlight the weakness of the experimental design in this manuscript.
- The DNA-binding assays are not physiologically relevant because they do not use DNA from the operator regulated by the candidate protein (why this was not explored in the revision is difficult to understand). The mobility shift observed at these high protein concentrations will result from non-specific binding. It is unsurprising that Cu(II) has an effect on DNA binding as it is added at such high concentrations relative to both protein and DNA so as to compete for DNA-binding with the protein (which binds weakly because there is no specific recognition site). The 10:1 ratio of Cu:CsoR is 10-times higher than needed as this class of proteins will show decreases in DNA-affinity in the presence of the correct metal at 1:1 stoichiometry. As indicated above, the authors need to consider alternative interpretations for their results rather than try to rationalise the results to fit the model.

The points raised above readily address the authors' own comments in the response as to their surprise at some of the results and their inconsistency with the model.

Even if the authors were to identify the correct metal to which the protein responds, there are still fundamental issues with experimental design and interpretation that would need to be addressed to indicate any link between the protein and chemotaxis.

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Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

*This report contains two parts. In the first part, several experiments were carried out to show that CsoR binds to CheA, inhibits CheA phosphorylation, and impairs *P. putida* chemotaxis. The second part provides some evidence that CsoR is a copper-binding protein, binds to CheA in a copper-dependent manner, and regulates *P. putida* response to copper, a chemorepellent. Based on these results, a working model is proposed to describe how CsoR coordinates chemotaxis and resistance to copper in *P. putida*. While the second part of the study is relatively solid, there are some major concerns about the first part.*

Critiques:

*(1) The rigor from prior research is not clear. In addition to talking about other bacterial chemotaxis, the Introduction should briefly summarize previous work on *P. putida* chemotaxis and copper resistance.*

We summarized previous results on *P. putida* copper resistance and added those results to the introduction section of the revised manuscript. As for chemotaxis, most studies in *P. putida* focused on the sensing/responding of the bacteria to different chemical compounds and the methyl-accepting chemotaxis proteins (MCPs) involved in the sensing, which is not relevant to the main content of this study. The component of the chemotaxis system in *P. putida* is similar to that in *E. coli*, and the signaling mechanism is presumably similar.

*(2) The rationale for identifying those CheA-binding proteins is vague. CheA has been extensively studied and its functional domains (P1 to P5) have been well characterized. Compared to its counterparts from other bacteria, does *P. putida* CheA contain a unique motif or domain? Does CsoR bind to other bacterial CheAs or only to *P. putida* CheA?*

The original purpose of the pull-down assay was to detect the interaction between CheA and c-di-GMP metabolizing enzymes, which was another project. However, we ignored that most c-di-GMP metabolizing enzymes were membrane proteins, and we made a mistake by using whole-cell lysate in the pull-down experiment. Thus, we failed to identify c-di-GMP metabolizing enzymes in “target” proteins of the pull-down assay. However, we found several novel “target” proteins in the pull-down assay. We wondered about the function of these proteins and the physiological roles of the interaction between CheA and these proteins, which was the primary purpose of this study. Although the function of CheA has been well characterized, most previous results focused on the role of CheA in chemotaxis, and its role in other bacterial processes was poorly studied. To extend our knowledge about CheA, we analyzed the results of the pull-down assay and decided to test the interaction between CheA and identified proteins, as well as the physiological roles of the interaction.

BLAST results showed that the CheA of *P. putida* shared 41.12% sequence similarity with the CheA of *E. coli*, and the CheA of *P. putida* had a similar domain pattern to those CheAs from other bacteria. To test whether CsoR_{*P. putida*} interacted with CheA from other bacteria, we performed a BTH assay to investigate the interaction between CsoR_{*P. putida*} and eight CheAs, including CheA from *E. coli*, CheA from *A. caldus*, CheA from *B. diazoefficiens*, CheA from *B. subtilis*, CheA from *L. monocytogenes*, CheA from *P. fluorescens*, CheA from *P. syringae*, and CheA from *P. stutzeri*. As shown in the following Fig. 1, CsoR_{*P. putida*} could interact with CheA from *A. caldus*, *B. subtilis*, *L. monocytogenes*, *P. fluorescens*, *P. syringae*, and *P. stutzeri*. Besides, among these strains, *cheA* and *csoR* coexist in *A. caldus*, *B. diazoefficiens*, *B. subtilis*, *L.*

monocytogenes, *P. fluorescens*, *P. syringae*, and *P. stutzeri*. We previously tested the interaction of the two proteins from these bacterial species. The results showed that the CheA-CsoR interaction existed between proteins from *A. caldus*, *B. subtilis*, *P. syringae*, and *P. stutzeri* (Fig. 7 in the manuscript). However, CheA and CsoR from *B. diazoefficiens*, *L. monocytogenes*, and *P. fluorescens* showed no apparent interaction (Fig. 7 in the manuscript). These results suggested that unique amino acid sequences in the two proteins might be required to achieve interaction.

(3) Line 133-136, "Collectively, using pull-down, BTH, and BiFC assays, we identified 16 new CheA-interacting proteins in *P. putida*." It is surprising that so many proteins were identified but none of them were chemotaxis proteins, in particular those known to interact with CheA, such as CheW, CheY and CheZ, which raises a concern about the specificity of these methods. BTH and BiFC often give false-positive results and thus should be substantiated by other approaches such as co-IP, surface plasmon resonance (SPR), or isothermal titration calorimetry (ITC) along with mutagenesis studies.

The response regulator CheY and the phosphatase CheZ (two proteins known to be associated with CheA) were identified in the pull-down assay (Table S1), and the two proteins showed high Log₂(fold change) values, indicating that they were obtained in the pull-down assay with high amount in the experimental group and low amount in the control group. Our study aimed to identify new CheA-interacting proteins; thus, the two proteins (CheY and CheZ) were not included in subsequent investigations. The CheA-interacting proteins were initially obtained through an *in vitro* assay (pull-down), followed by an *in vivo* assay (BTH and BiFC) to test the interaction further. Only proteins that showed positive results in all three assays were considered trustworthy CheA-interacting proteins and kept for further study.

(4) Line 147-149, "Fig. 2a, five strains (WT+*pcsoR*, WT+*pispG*, WT+*pnfuA*, WT+*pphaD*, and WT+*pPP_1644*) displayed smaller colony than the control strain (WT+*pVec*), indicating a weaker chemotaxis ability in these five strains." If copper is a chemorepellent, these strains should swim away from high concentrations of copper; thus, the sizes of colonies couldn't be used to measure this response. In the cited reference (reference 29), bacterial response to phenol was measured using a response index (RI).

Except for CsoR, the rest of the CheA-interacting proteins had no direct connection with copper and were involved in different processes (Table S1). A reasonable speculation is that these proteins involved in different processes can integrate signals from specific processes into chemotaxis by regulating CheA autophosphorylation, leading to better regulation of chemotaxis according to intracellular physiological state. We used semisolid nutrient agar plates to test and compare bacterial chemotaxis ability. In a fixed attractant/repellent gradient, chemokine, such as copper, can lead to two subpopulations traveling at different speeds, with the slower one being held back by the chemokinetic drift. In the case of semisolid plate migration, bacteria with chemotaxis ability formed large colonies by generating their gradient by consuming nutrients/producing toxic metabolic waste and following attractant/repellent gradients leading outward from the colony origin (Cremer et al., 2019. Nature 575:658–663). The observation of successive sharp circular bands (rings) progressing outward from the inoculation point was taken to confirm the chemotaxis genotype, and mutants without chemotaxis spread out uniformly and formed a small colony (Wolfe and Berg, PNAS. 1989, 86:6973-6977). In our experiment, we were unsure about the signals/chemokines of each target protein, so we could not design a fixed attractant/repellent gradient. Besides, all target proteins interacted with CheA, which is a crucial factor in chemotaxis, and we assume that these proteins would affect chemotaxis under overexpression conditions. Thus, we used semisolid nutrient plates to test and compare bacterial chemotaxis ability.

(5) Figures 2 and 3 show both CsoR and PhaD bind to CheA and inhibit CheA autophosphorylation. Do these two proteins share any sequence or structural similarity? Does PhaD also bind to copper? Otherwise, it is difficult to understand these results.

Thanks a lot. This is an enlightening comment. CsoR is a protein with a size of 10.8 kDa, and PhaD is 23.1 kDa. Because of the difference in size, we took it for granted that the two proteins were not similar. We recently compared their sequence on NCBI BLAST. Although both CsoR and PhaD are transcriptional regulators and interact with CheA, they have no significant sequence similarity. In terms of protein structure, we predicted their structures using AlphaFold. The results showed that CsoR consisted of three α -helices and PhaD consisted of nine α -helices (new Fig. S5a and S5b in the manuscript). We further compared their structure using Pymol but found no significant similarity between the two proteins (new Fig. S5c in the manuscript).

PhaD is a TetR family transcriptional regulator located adjacent to the genes involved in PHA biosynthesis, and it behaves as a carbon source-dependent activator of the *pha* cluster related to polyhydroxyalkanoates (PHAs) biosynthesis (de Eugenio et al., Environ Microbiol. 2010, 12:1591-1603; Tarazona et al., Environ Microbiol. 2020, 22:3922-3936). Bacterial PHAs are isotactic polymers synthesized under unfavorable growth conditions in the presence of excess carbon sources. PHAs are critical in central metabolism, acting as dynamic carbon reservoirs and reducing equivalents (Gregory et al., Trends Mol Med. 2022, 28:331-342). The interaction between PhaD and CheA leads us to speculate that there might be some connection between PHA synthesis and bacterial chemotaxis. For example, chemotaxis helps bacteria move towards specific carbon sources that favor PHA synthesis, and the interaction between PhaD and CheA weakens chemotaxis, causing bacteria to linger in areas rich in these carbon sources. This is an interesting hypothesis worth testing in the future.

(6) Line 195-196, "CsoR/PhaD had no apparent influence on the phosphate transfer between CheA and CheY". CheA controls bacterial chemotaxis through CheY phosphorylation. If this is true, how do CsoR and PhaD affect chemotaxis?

During the autophosphorylation assay, CheA was mixed with CsoR/PhaD and incubated for about 10 min before adding [32 P]ATP[γ P]. Thus, the effect of CsoR/PhaD on CheA autophosphorylation happened through the assay, and a significant inhibition effect was observed in the final result. Regarding transphosphorylation, CheA was mixed with ATP and incubated for about 30 min, at which time the autophosphorylation of CheA happened. Then, CsoR/PhaD and CheY were added to the phosphorylated CheA to investigate transphosphorylation. CsoR and PhaD affected chemotaxis via inhibiting CheA autophosphorylation, which was a crucial step in chemotaxis signaling, and the decrease in CheA autophosphorylation caused decreased chemotaxis.

(7) Figure 3 shows that CsoR/PhaD bind to CheA through P1, P3, and P4. This result is intriguing. All CheA proteins contain these three domains. If this is true, CsoR/PhaD should bind to other bacterial CheAs too. That said, this experiment is premature and needs to be confirmed by other approaches.

As replied to comment (2) above, we performed a BTH assay to investigate whether CsoR_{p. putida} interacts with CheA from other bacterial species. The results revealed that CsoR_{p. putida} interacted with CheA from *A. caldus*, *B. subtilis*, *L. monocytogenes*, *P. fluorescens*, *P. syringae*, and *P. stutzeri*, but not with CheA from *E. coli* and *B. diazoefficiens*. This result suggested that CheA-CsoR interaction required specific/unique amino acid sequence patterns in the two proteins, and similar domain composition alone was insufficient.

(8) Figure 5, does PhaD contain these three residues (C40, H65, and C69)? If not, how does PhaD inhibit CheA autophosphorylation and chemotactic response to copper?

No, there is no significant sequence similarity between PhaD and CsoR, and PhaD contains none of the three residues of CsoR (C40, H65, and C69). The size of the two proteins is also quite different (CsoR 10.8 kDa, PhaD 23.1 kDa). The structure alignment also revealed no apparent similarity between the predicted structures of PhaD and CsoR (new Fig. S5c in the manuscript). Nevertheless, CsoR and PhaD interacted with CheA through its P1, P3, and P4 domains. It is interesting how the two proteins interacted with CheA, but we currently have no answer.

(9) Does deletion of *cosR* or *cheA* have any impact on *P. putida* resistance to high concentrations of copper?

No, deletion of *cosR/cheA* had no noticeable impact on *P. putida*'s resistance to high concentrations of copper. We performed a growth assay to test the effect of CsoR and CheA on copper resistance under both liquid and solid medium conditions. The copper concentration was set at 0, 200, 500, 1000 μ M. With the increase of copper concentration, the growth of bacteria was gradually inhibited, but the growth trends of *csoR* mutant, *cheA* mutant, and complementary strains were similar to that of the wild-type strain (new Fig. S6b and S6c in the manuscript). We speculated that this might be attributed to CsoR being a repressor and inhibiting gene expression in the absence of copper. When copper existed, the inhibitory effect of CsoR was relieved, which is the same as that in the *csoR* mutant. Besides, although deletion of *cosR* led to a slight increase (about 1.3-fold) in the expression of copper resistance genes (Fig. 4b in the manuscript), its effect on gene expression was much weaker than its homologous protein in other bacterial species. In *M. tuberculosis*, *B. subtilis*, *C. glutamicum*, *L. monocytogenes*, and *S. aureus*, deletion of *csoR* resulted in an about 10-fold increase in the expression of target genes in the absence of copper. This difference might be attributed to several vital regulators that activated the expression of copper-resistance genes in response to copper in *P. putida*, such as CueR and CopR (Adaikkalam and Swarup, Microbiology. 2002, 148:2857-2867; Hofmann et al., Int J Mol Sci, 2021, 22:2050; Quintana et al., J Biol Chem, 2017, 292:15691-15704). CueR positively regulated the expression of *cueA*, encoding a copper-transporting P1-type ATPase that played a crucial role in copper resistance. CopR was essential for expressing several genes implicated in cytoplasmic copper homeostasis, such as *copA-II*, *copB-II*, and *cusA*. The existence of these positive regulators makes the function of CosR a secondary or even dispensable insurance in the expression of copper-resistance genes. Consistent with this, there is no CosR homolog in *P. aeruginosa*, and copper homeostasis is mainly controlled by CueR and CopR.

Reviewer #2 (Public Review):

This manuscript focuses on the apparent involvement of a proposed copper-responsive regulator in the chemotactic response of *Pseudomonas putida* to Cu(II), a chemorepellent. Broadly, this area is of interest because it could provide insight into how soil microbes mitigate metal stress. Additionally, copper has some historical agricultural use as an antimicrobial, thus can accumulate in soil. The manuscript bases its conclusions on an in vitro screen to identify interacting partners of CheA, an essential kinase in the *P. putida* chemotaxis-signaling pathway. Much of the subsequent analysis focuses on a regulator of the CsoR/RcnR family (PP_2969).

Weaknesses:

The data presented in this work does not support the model (Figure 8). In particular, PP_2969 is linked to Ni/Co resistance, not Cu resistance. Further, it is not clear how the

putative new interactions with CheA would be integrated into diverse responses to various chemoattract/repellents. These two comments are justified below.

Thanks a lot for all these comments. Before designing experiments to explore the function of PP_2969, we found three clues: (i) its sequence showed 38% similarity to the copper-responsive regulator CsoR of *M. tuberculosis*, and the three conserved amino acids essential for copper-binding were conserved in PP_2969; (ii) it located next to a Ni²⁺/Co²⁺ transporter (PP_2968) on the genome; (iii) a previous report revealed that PP_2969 (also named MreA) expression increased during metal stress, and overexpression of PP_2969 in *P. putida* and *E. coli* led to metal accumulation (Zn, Cd, and Cr) (Lunavat et al., Curr Microbiol. 2022, 79:142). These clues indicate that the function of PP_2969 is related to metal-binding, but it remains to be explored which metal(s) PP_2969 binds to. Thus, we played MST assay to test the interaction between PP_2969 and metals, including copper (Cu²⁺), zinc (Zn²⁺), nickel (Ni²⁺), cobalt (Co²⁺), cadmium (Cd²⁺), and magnesium (Mg²⁺). The result showed that PP_2969 was bound to three metal ions (Cu²⁺, Zn²⁺, Ni²⁺), and the binding to Cu²⁺ was the strongest. Besides, the EMSA assay revealed that Cu²⁺/Ni²⁺/Zn²⁺ inhibited the interaction between PP_2969 and promoter DNA, and Cu²⁺ showed the most substantial inhibitory effect at the same concentration. These results suggested that PP_2969 was mainly bound to Cu²⁺, followed by Zn²⁺ and Ni²⁺. To further test whether PP_2969 functioned as a metal-responsive repressor and which metal resistance was related to its target gene, we constructed a PP_2969 deletion mutant and complementary strain and performed a qPCR assay to compare the expression of metal resistance-related genes. 14 metal-resistant-related genes were chosen as targets. The results showed that PP_2969 deletion led to a weak but significant increase (about 1.3-fold) in expression of 10 genes, including three copper-resistance genes (*copA-I*, *copA-II*, and *copB-II*), one nickel-resistance gene (*nikB*), two cadmium-resistance genes (*cadA-I* and *cadA-III*), one cobalt-resistance gene (*cbtA*), and three multiple metal-resistance genes (*czcC-I*, *czcB-II*, and PP_0026) (Fig. 4b, Fig. S5a in the manuscript). Meanwhile, complementation with a multicopy plasmid containing the PP_2969 gene decreased the gene expression in Δ PP_2969. Although PP_2969 regulated the expression of multiple metal resistance genes, it showed the most robust binding to Cu²⁺. Thus, we considered its primary function as a Cu²⁺-responsive regulator.

As for the second comment, “How would the putative new interactions with CheA be integrated into diverse responses to various chemoattract/repellents?”, We have some speculations based on our results and previous reports. For example, PP_2969 interacted with CheA and decreased its autophosphorylation activity, and copper inhibited the interaction between CheA and PP_2969. In the absence of copper, PP_2969 binds to promoters to inhibit the expression of copper resistance genes, and it also binds to CheA to inhibit its autophosphorylation, resulting in lower chemotaxis. When the bacteria move to an area of high copper concentration, PP_2969 binds to copper and falls off the DNA promoter, leading to higher expression of copper resistance genes. Meanwhile, copper-binding of PP_2969 decreases its interaction with CheA, increasing CheA autophosphorylation promoting chemotaxis, and bacteria swim away from the high copper concentration. Another attractive target protein is PhaD, a TetR family transcriptional regulator located adjacent to the genes involved in PHA biosynthesis, and it behaves as a carbon source-dependent activator of the *pha* cluster related to polyhydroxyalkanoates (PHAs) biosynthesis (de Eugenio et al., Environ Microbiol. 2010, 12:1591-1603; Tarazona et al., Environ Microbiol. 2020, 22:3922-3936). Bacterial PHAs are isotactic polymers synthesized under unfavorable growth conditions in the presence of excess carbon sources. PHAs are critical in central metabolism, acting as dynamic carbon reservoirs and reducing equivalents (Gregory et al., Trends Mol Med. 2022, 28:331-342). The interaction between PhaD and CheA leads us to speculate that there might be some connection between PHA synthesis and bacterial chemotaxis. For example, chemotaxis helps bacteria move towards particular carbon sources that favor PHA synthesis; the regulator PhaD activates the genes related to PHA synthesis. Meanwhile, the interaction

between PhaD and CheA weakens chemotaxis, causing bacteria to linger in areas rich in these carbon sources. Collectively, we speculate that by interacting with CheA and modulating its autophosphorylation, target proteins such as CsoR/PhaD integrate signals from their original process pathway into chemotaxis signaling.

PP_2969

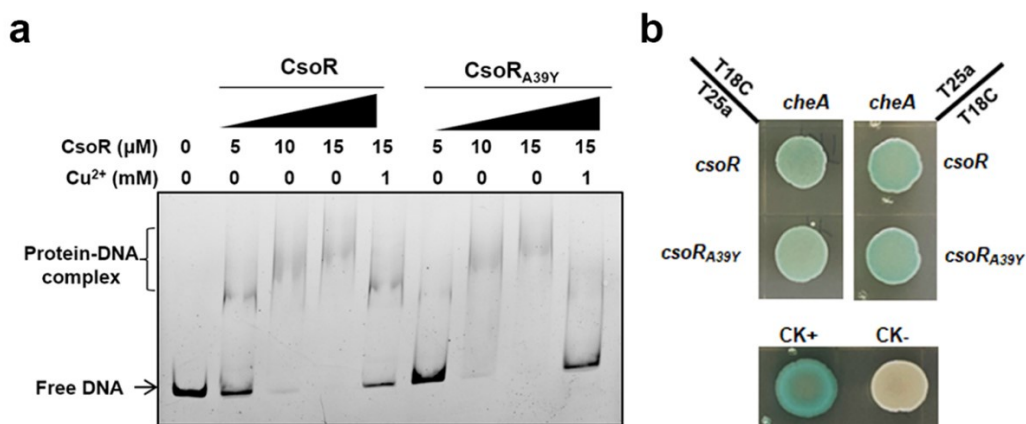
(1) The authors present a sequence alignment (Figure S5) that is the sole basis for their initial assignment of this ORF as a CsoR protein. There is a conservation of the primary coordinating ligands (highlighted with asterisks) known to be involved in Cu(I) binding to CsoR (ref 31). There are some key differences, though, in residues immediately adjacent to the conserved Cys (the preceding Ala, which is Tyr in the other sequences). The effect of these changes may be significant in a physiological context.

We constructed a point mutation in PP_2969 by replacing the Ala residue before the conserved Cys with a Tyr (CsoR_{A39Y}) and then analyzed the effect of this mutation on CsoR. As shown in Author response image 1a, CsoR_{A39Y} showed similar promoter-binding ability as the wild-type CsoR and the presence of Cu²⁺ abolished the interaction between CsoR_{A39Y} and DNA, suggesting that the A39 residue in PP_2969 was not essential for the DNA-binding and Cu²⁺-binding abilities. Besides, CsoR_{A39Y} interacted with CheA as the wild-type CsoR did (Author response image 1b), indicating that the Ala39 residue was not required to interact with CheA.

The CsoR from *B. subtilis* has a Tyr before the conserved Cys, which is the same as other sequences, and the BTH result showed that interaction existed between CsoR and CheA from *B. subtilis* (Fig. 7 in the manuscript).

Author response image 1.

The effect of CsoR point mutation (CsoR_{A39Y}) on the DNA-binding and Cu²⁺-binding abilities of CsoR. (a) Analysis for interactions between CsoR/CsoR_{A39Y} and *copA-I* promoter DNA using EMSA. The concentrations of CsoR/CsoR_{A39Y} and Cu²⁺ added in each lane are shown above the gel. Free DNA and protein-DNA complexes are indicated. (b) The interaction between CsoR/CsoR_{A39Y} and CheA was tested by BTH. Blue indicates protein-protein interaction in the colony after 60 h of incubation, while white indicates no protein-protein interaction. CK+ represents positive control, and CK- represents negative control.



(2) The gene immediately downstream of PP_2969 is homologous to *E. coli* RcnA, a demonstrated Ni/Co efflux protein, suggesting that P2969 may be Ni or Co responsive. Indeed PP_2970 has previously been reported as Ni/Co responsive (J. Bact 2009

doi:10.1128/JB.00465-09). The host cytosol plays a critical role in determining metal response, in addition to the protein, which can explain the divergence from the metal response expected from the alignment.

Correction: The gene immediately upstream (not downstream) of *PP_2969* (the ID is *PP_2968*, not *PP_2970*) is homologous to *E. coli* *RcnA*, a demonstrated Ni/Co efflux protein. The previous JBact study (J. Bact 2009 doi:10.1128/JB.00465-09) named *PP_2968* as *MrdH*, and *mrdH* disruption led to sensitivity to cadmium, zinc, nickel, and cobalt, but not copper. Their results also revealed that *MrdH* was a broad-spectrum metal efflux transporter with a substrate range including Cd^{2+} , Zn^{2+} , and Ni^{2+} . However, the role of *MrdH* in Cu^{2+} efflux was not tested. Commonly, metal efflux transporter has a broad substrate spectrum, allowing transporters to influence bacterial resistance to a variety of metals (Munkelt et al., J Bacteriol. 2004, 186:8036-8043; Grass et al., J Bacteriol. 2005, 187:1604-1611; Nies et al., J Ind Microbiol. 1995, 14:186-199; Kelley et al., Metallomics. 2021, 13:mfaa002). Our results showed that *PP_2969* bound to Cu^{2+} , Zn^{2+} , and Ni^{2+} under our experimental conditions, and *CsoR* regulated the expression of genes related to Cu^{2+} , Zn^{2+} , and Ni^{2+} resistance, indicating that *CsoR* was involved in resistance to these metals. But the binding of *CsoR* to Cu^{2+} was the strongest, and Cu^{2+} showed the most substantial inhibitory effect on *CsoR*-DNA interaction. Thus, we considered its primary function as a Cu^{2+} -responsive regulator.

(3) The previous JBact study also explains the lack of an effect (Figure 5b) of deleting PP_2969 on copper-efflux gene expression (copA-I, copA-II, and copB-II) as these are regulated by CueR not PP_2969 consistent with the previous report. Deletion of CsoR/RcnR family regulator will result in constitutive expression of the relevant efflux/detoxification gene, at a level generally equivalent to the de-repression observed in the presence of the signal.

We performed qPCR to test the effect of *PP_2969* on gene expression, and we chose 14 target genes, including copper-resistance genes, nickel-resistance genes, zinc-resistance genes, cadmium-resistance genes, and cobalt-resistance genes. The results showed that *PP_2969* deletion led to a weak but significant increase (about 1.3-fold) in the expression of 10 genes (Fig. 4b, new Fig. S5a in the manuscript), and complementation with a multicopy plasmid containing *PP_2969* gene decreased the gene expression in ΔPP_2969 . We were confused about these results. Why was the effect of *PP_2969* on gene expression so weak? Did we pick the wrong target genes? In other bacteria, deletion of *csoR* led to an about ten-fold increase in gene expression, generally equivalent to the de-repression observed in the presence of metal. Thus, to further identify target genes, we performed RNA-seq to compare the gene expression in WT and ΔPP_2969 without copper. The result surprised us because no gene expression levels changed more than two-fold (data not shown). This result might be attributed to several vital regulators that activated the expression of metal-resistance genes in response to metal in *P. putida*, such as *CueR* and *CopR* (Adaikkalam and Swarup, Microbiology. 2002, 148:2857-2867; Hofmann et al., Int J Mol Sci, 2021, 22:2050; Quintana et al., J Biol Chem, 2017, 292:15691-15704). *CueR* positively regulated the expression of *cueA*, encoding a copper-transporting P1-type ATPase that played a crucial role in copper resistance. *CopR* was essential for expressing several genes implicated in cytoplasmic copper homeostasis, such as *copA-II*, *copB-II*, and *cusA*. The existence of these positive regulators might make the function of *CosR* a secondary or even dispensable insurance in the expression of copper-resistance genes. Consistent with this, there is no *CosR* homolog in *P. aeruginosa*, and copper homeostasis is mainly controlled by *CueR* and *CopR*.

(4) Further, CsoR proteins are Cu(I) responsive so measuring Cu(II) binding affinity is not physiologically relevant (Figures 5a and S5b). The affinities of demonstrated CsoR proteins are 10-18 M and these values are determined by competition assay. The MTS assay and resulting affinities are not physiologically relevant.

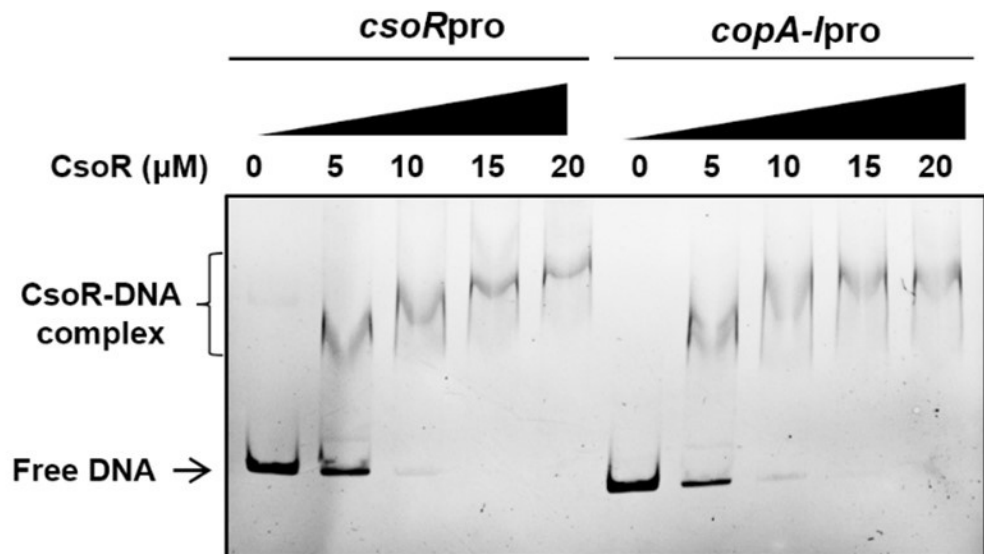
Thank you for this enlightening comment. This question also confused us during our experiment. The first study on CsoR from *Mycobacterium tuberculosis* showed that CsoR bound a single-monomer mole equivalent of Cu(I) to form a trigonally coordinated complex, and that was a convincing result from protein structure analysis (Liu et al., Nat Chem Biol. 2007, 3:60-68). They further revealed that the presence of Cu(I) in the EMSA assay abolished the DNA-binding ability of CsoR, but the impact of Cu(II) was not tested. Besides, their results also showed that adding CuCl₂ in the medium induced the expression of the *cso* operon involved in copper resistance. Perhaps Cu(II) converted to Cu(I) and then bound to CsoR in bacterial cells. Later studies in diverse bacterial species (including *Listeria monocytogenes*, *Corynebacterium glutamicum*, *Deinococcus radiodurans*, and *Thermus thermophilus*) showed that *in vitro* assays with Cu(II) abolished the DNA-binding ability of CsoR, indicating that CsoR bound to both Cu (I) and Cu(II) (Corbett et al., Mol Microbiol. 2011, 81:457-472; Teramoto et al., Biosci Biotechnol Biochem. 2012, 76:1952-1958; Zhao et al., Mol Biosyst. 2014, 10:2607-2616; Sakamoto et al., Microbiology. 2010, 156:1993-2005). Here, our results from *in vitro* assays (MST and EMSA) showed that CsoR bound to Cu(II) and Cu(II) affected the interaction between CsoR and promoter DNA. Compounds containing Cu(I) are poorly soluble in water and easily oxidized by Cu(II). DTT can reduce Cu(II) to Cu(I) (Krzew et al., J Inorg Biochem. 2001, 84:77-88). To test whether Cu(I) bound to CsoR and affected its DNA-binding ability, we recently performed an EMSA assay with the addition of CuCl₂/DTT/CuCl₂+DTT. As shown in Fig. 4d, the addition of DTT (0.1 and 1 mM) decreased CsoR-DNA interaction in the presence of 0.2 mM CuCl₂, while the addition of DTT alone had no apparent influence on CsoR-DNA interaction, indicating that DTT enhanced the inhibition of CuCl₂ on CsoR-DNA interaction, and the Cu(I) converted from Cu(II) by DTT had stronger inhibitory effect than Cu(II) on CsoR-DNA interaction. Together, these results suggested that CsoR bound to Cu(I) more strongly than it bound to Cu(II). We have added these results to the new version of manuscript.

(5) The DNA-binding assays are carried out at protein concentrations well above physiological ranges (Figures 5c and d, and S5c, d). The weak binding will in part result from using DNA sequences upstream of the *copA* genes and not from PP_2970.

We performed *the vitro* DNA-binding assay several times, and the lowest CsoR concentration used to obtain a shifted band was about 3 μM, and a higher concentration (15 μM) caused total DNA binding. Thus, we used the concentration of 15 and 20 μM to test the effect of metal on protein-DNA interaction in the assay. We also realized that these concentrations were above physiological ranges. We considered that the *in vitro* DNA-binding assay was only a mimic of the *in vivo* process, and the extracellular physiological conditions in EMSA might restrict the activity of CsoR. Besides, we recently performed EMSA to investigate the interaction between CsoR and its own promoter (*csoRpro*). As shown in Author response image 2, CsoR bound to *csoRpro* with a similar intensity to that it bound to *copA-Ipro*. Thus, the weak binding was not caused by the promoter used in the assay.

Author response image 2.

The binding of CsoR to its own promoter (*csoRpro*) and *copA-I* promoter (*copA-1pro*) in EMSA. The concentrations of CsoR added in each lane are shown above the gel. Free DNA and CsoR-DNA complex are indicated.



CheA interactions

(1) *There is no consideration given to the likely physiological relevance of the new interacting partners for CheA.*

Thank you for this comment. The initial purpose of this research was to identify new CheA-interacting proteins to broaden our knowledge of CheA and bacterial chemotaxis. Thus, we are currently focusing on the effect of the interaction on CheA and chemotaxis and trying to find the link between different processes and bacterial chemotaxis. We infer that the interaction between these new interacting partners and CheA can integrate signals from different pathways into the chemotaxis signaling pathway so that bacteria can better sense and adapt to different environments. Besides, the other role of the interaction, which is the influence of CheA on these new interacting partners, is also an exciting question that remains to be answered. Among the 16 new CheA-interacting proteins, five showed significant influence on chemotaxis, and the remaining 11 proteins had no obvious impact on chemotaxis (Fig. 2a in the manuscript). CsoR and PhaD inhibited CheA autophosphorylation, and here we focused on the effect of CsoR on chemotaxis. We also investigated the impact of CheA on CsoR, such as gene regulation and copper resistance. However, the results showed that CheA had no obvious influence on these functions of CsoR. The interactions between CheA and these proteins may be physiologically biased, with some interactions affecting the function of CheA and others mainly affecting the function of partners. Future studies on the function of these new CheA-interacting proteins and the role of CheA in regulating their functions would further expand our knowledge of CheA.

(2) *How much CheA is present in the cell (copies) and how many copies of other proteins are present? How would specific responses involving individual interacting partners be possible with such a heterogenous pool of putative CheA-complexes in a cell? For PP_2969, the affinity reported (Figure 5A) may lay at the upper end of the CsoR concentration range (for example, CueR in Salmonella is present at ~40 nM).*

Thank you for this insightful comment. We don't know the copy number of CheA and other proteins in the cell. We were also initially surprised and felt skeptical about the reliability of CheA interaction with so many proteins. CheA interacts with CheY, CheW, and CheB in the classical chemotaxis pathway. This study found 16 new CheA-interacting proteins using pull-

down assay and subsequent analysis. Moreover, in another unpublished result, we found that CheA interacted with eight c-di-GMP-metabolizing proteins, and CheA transferred the phosphate group to one of them. Together, it seemed that CheA could interact with at least 27 proteins. With such a heterogeneous pool of CheA-complexes, performing a specific response seemed difficult. However, several previous studies have reported the example of one protein interacting with dozens of proteins. For example, the c-di-GMP effector LapD in *Pseudomonas fluorescens* and *Pseudomonas putida* can interact with a dozen different c-di-GMP-metabolizing proteins (Giacalone et al., mBio. 2018, 9:e01254-18; Nie et al., Mol Microbiol. 2024, 121:1-17.) In *Escherichia coli*, a subset of DGCs and PDEs operated as central interaction hubs in a larger “supermodule” by interacting with dozens of proteins (Sarenko et al., mBio. 2017, 8:e01639-17). We infer that the expression of different CheA-interacting proteins might happen at different growth stages or under different conditions, and their interaction with CheA under that stage/condition changed bacterial chemotaxis or the process in which the target protein was involved.

(3) The two-hybrid system experiment uses a long growth time (60 h) before analysis. Even low LacZ activity levels will generate a blue color, depending upon growth medium (see doi: 10.1016/0076-6879(91)04011-c). It is also not clear how Miller units can be accurately or precisely determined from a solid plate assay (the reference cited describes a protocol for liquid culture).

We didn't observe a blue color on the colony after 60 h growth on a plate under our experimental conditions. The BTH experiment was described as follows: After transforming the two plasmids into *E. coli* BTH101 cells, the plates containing transformants were placed at 28° for 48 h, at which time the colonies of the transformants were big enough to be picked up and incubated in a liquid medium for 24 h at 28°. Then, 5 µL of the culture was spotted onto an LB agar plate supplemented with antibiotics, X-gal, and IPTG and incubated for 60 h at 28° before taking the photos. After the photos were taken, the bacteria on the plate were scraped off and resuspended with buffer, and then the LacZ activity of the bacteria was tested. According to our experience, culture at 28°(lower than 30°) is a critical condition, and we have not observed false positives in BTH assays under this condition.

Reviewer #1 (Recommendations For The Authors):

In addition to genetic and biochemical approaches, structural studies should be conducted to elucidate the molecular interaction between CheA and CsoR with/without copper.

It would be more logical to first establish the role of CsoR in copper regulation and chemotaxis (the second part of this report) and then investigate its underpinning mechanism (the first part).

Thank you for these recommendations. Structural analysis can reveal more details about the molecular mechanism of CheA-CsoR interaction, but we currently don't have sufficient experimental conditions for such structural analysis.

As for the presentation logic of the results, we wrote the manuscript following the sequence of experiments. Firstly, screening of CheA interacting proteins (pull-down assay) was conducted, and then the influence of interacting proteins on the chemotaxis of strains and CheA autophosphorylation activity was detected. Based on these results, we obtained two proteins, CsoR and PhaD, and decided to go deeper into the function of CsoR and its effect on chemotaxis. We considered that this writing logic reflected our research design better and could also lay a foundation for future exploration of the functions of other interacting proteins and the physiological significance of interactions.

Reviewer #2 (Recommendations For The Authors):

A huge amount of effort has gone into this work.

It would be good to see at least one of the newly identified interactions turn out to be physiologically relevant.

The experimental tools appear to be available to do this, but it is critical to consider how these tools can lead to attempts to prove rather than test and possibly refute a model or hypothesis. In particular, please consider some of the comments about the physiological relevance of affinities when generating models.

Thank you for these recommendations. Our study aimed to screen new interacting proteins of CheA and explore how new interacting proteins affect CheA activity and bacterial chemotaxis, thereby broadening our understanding of chemotaxis. However, the impact of each protein-protein interaction has two sides: the influence of A to B and B to A. During experimental design, we focused more on the influence of identified interacting proteins on CheA function and chemotaxis but paid less attention to the function of interacting proteins and the influence of the interaction on their function. Moreover, our study found that the influence of protein-protein interaction was biased. In the interaction between CsoR and CheA, CsoR mainly affected the function of CheA and then affected the chemotaxis, while CheA had no significant effect on the function of CsoR. This might be attributed to the weak effect of CsoR in regulating metal resistance in *P. putida*, and we speculated that this interaction was more about favoring the sensing and avoiding metal stress. In addition, we planned to explore the interaction between CheA and another interacting protein (PhaD) in the future, reveal the effect of the interaction on PhaD function (regulation of PHAS synthesis in bacteria), and explore the effect of the interaction on CheA function and chemotaxis, to find out whether the association existed between PHAS anabolism and bacterial chemotaxis. Besides, for those proteins that did not have significant effects on CheA autophosphorylation and bacterial chemotaxis, we speculated that CheA might affect their function/activity through interactions, which meant that the physiological effects of the interaction mainly reflected through the interacting protein rather than CheA. These are speculations that need to be tested by experiments.

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